EXPERIMENTAL CELL RESEARCH

Published under the auspices of

THE INTERNATIONAL SOCIETY FOR CELL BIOLOGY

EDITORS:

TORBJÖRN CASPERSSON • HONOR B. FELL • JOHN RUNNSTRÖM

FRANCIS O. SCHMITT • PAUL WEISS • RALPH W. G. WYCKOFF

VOLUME 3 • FEBRUARY 1952 • NUMBER 1

ACADEMIC PRESS INC., PUBLISHERS, NEW YORK, N.Y.

EXPERIMENTAL CELL RESEARCH

EDITORS:

T. Caspersson, Department of Cell Research, Karolinska Inst., Stockholm 60, Sweden
Honor B. Fell, Strangeways Laboratory, Cambridge, England
J. Runnström, The Wenner-Gren Institute for Experimental Biology, Stockholm, Sweden
Francis O. Schmitt, Massachusetts Institute of Technology, Cambridge 39, Massachusetts
Paul Weiss, University of Chicago, Chicago 37, Illinois
Ralph W. G. Wyckoff, Laboratory of Physical Biology, National Institute of Health, Bethesda, Maryland.

EXECUTIVE COMMITTEE:

T. Caspersson, Honor B. Fell, J. Runnström and J. F. Danielli, King's College, London, W. C. 2, England.

Papers originating from Europe should be submitted to Dr. Honor B. Fell or to the Editorial Office, Norrtullsgatan 16, Stockholm Va.

Papers from the United States and South America should be sent to one of the American editors.

ADVISORY BOARD:

H. Bauer (Wilhelmshafen), W. Bloom (Chicago), J. Brachet (Bruxelles), F. Buchthal (Copenhagen), A. Buzzati-Traverso (Verbania Pallanza), R. Chambers (New York), E. Chargaff (New York), J. F. Danielli (London), C. D. Darlington (London), J. N. Davidson (Glasgow), M. Demerec (Cold Spring Harbor, N. Y.), J. Engelbreth-Holm (Copenhagen), E. Fauré-Frémiet (Paris), A. Fischer (Copenhagen), A. Frey-Wyssling (Zürich), N. Fries (Uppsala), R. J. Gautheret (Paris), L. Geitler (Wien), G. Gomori (Chicago), A. Haddow (London), E. Hammarsten (Stockholm), B. Hanström (Lund), E. Newton Harvey (Princeton, N. J.), L. V. Heilbrunn (Philadelphia, Pa.), G. C. Heringa (Amsterdam), A. Hollaender (Oak Ridge, Tenn.), H. Holmgren (Stockholm), H. Holter (Copenhagen), J. Holtfreter (Rochester, N. Y.), P. Koller (London), F. Lehmann (Bern), P. L'Héritier (Paris), G. Levi (Torino), P. E. Lindahl (Uppsala), G. Lindeberg (Uppsala), C. C. Lindegren (Carbondale, Ill.), R. J. Ludford (London), S. E. Luria (Bloomington, Ind.), A. Lwoff (Paris), A. Mirsky (New York), G. Montalenti (Naples), H. Okkels (Copenhagen), O. Olivo (Bologna), A. W. Pollister (New York), J. Randall (London), W. J. Schmidt (Giessen), F. Schrader (New York), W. Seifriz (Philadelphia, Pa.), T. M. Sonneborn (Bloomington, Ind.), J. M. Sosa (Montevideo), J. Spek (Rostock), W. M. Stanley (Berkeley, Calif.), E. Suomalainen (Helsingfors), E. L. Tatum (Stanford University, Calif.), T. Teorell (Uppsala), A. Tyler (Pasadena, Calif.), W. Wilbrandt (Bern).

Subscriptions

EXPERIMENTAL CELL RESEARCH

will be published in four issues annually at $15.00 per volume. The price of one issue is $4.00. Please send your subscription orders to Academic Press Inc., 125 East 23 St., New York 10, N.Y.

THE VISUALIZATION, BY MEANS OF PYRONIN, OF THE RNA-SYSTEM, INDICATING CYTOPLASMIC PROTEIN SYNTHESIS IN THE ANTERIOR PITUITARY OF THE GUINEA PIG

LEO ABOLINŠ

The Wenner-Grens Institute for Experimental Biology, University of Stockholm, Sweden

Received March 1, 1951

Whereas the determination of desoxyribonucleic acid in tissues by means of the reagent of Feulgen and Rossenbeck (23) has developed into an elaborated cytochemical method, the estimation of ribonucleic acid still involves some difficulties. It was Brachet (4–6) who found that it is actually the ribonucleic acid which avidly reacts with pyronin of the Unna-Pappenheim's methyl green-pyronin stain mixture. Another important contribution of Brachet was the introduction of the ribonuclease enzymatic digestion test and the RNA hot water-solubility control in the Unna-Pappenheim technique mentioned. By these contributions the usual staining procedure for basophilic substances in cells became a specific cytochemical method, the Brachet-Pappenheim RNA detection method. The investigations by Michaelis (41), Lison (39), Kurnick (34), Kurnick and Mirsky (35) and Pollister and Leuchtenberger (46) have subsequently greatly promoted the understanding of the mechanism of the action of dyes used in this method.[1]

To our knowledge, Celestino da Costa (17) was the first to discover the basophilic "filaments ergastoplasmiques" in both chromophile and chromophobe cells of the anterior pituitary (of the guinea pig). In chromophobe cells these fibrous masses were localized in various parts of the cytoplasm, mainly around the nuclei, at the cell periphery, or in form of calottes in the cytoplasm. In chromophile cells they mostly filled the entire cytoplasm. To judge from figures published by da Costa, and from our own experience, they may have been of a nucleic acid nature. This was already assumed by Desclin (21), who could observe similar formations in pituitaries of the rat and the guinea pig stained with stain mixtures of Dominici, May-Grünwald-Giemsa, and Unna-Pappenheim. Desclin also observed the disappearance of these formations after the treatment of sections with ribonuclease, thus being the first to disclose their RNA character. Afterwards Herlant (27) observed the RNA in anterior pituitaries of man, Dempsey and Wislocki

(19) in those of the rat, Tuchmann-Duplessis and Labat (56) in those of the mouse, Desclaux (20) further in those of the guinea pig, and finally Racadot (47) in those of cattle, sheep, and pigs. All these authors have proved the presence of RNA in sections of pituitaries cytochemically.

In a previous paper (1) dealing with the distribution of alkaline phosphatase in the anterior pituitary of the guinea pig, we also could confirm the occurrence of RNA, especially in acidophile cells of this gland. The researches chiefly concern the relatively little known mode of intimate localization of RNA in different cytological structures and the still confused question as to the occurrence of RNA in various cell types of the pituitary. Some observations on the localization of RNA are presented below.

MATERIAL AND METHODS

The procedure is indicated here only briefly. The pituitaries have been fixed in mixtures by Carnoy and Helly, in 95 per cent alcohol and in absolute acetone. The best results were obtained according to Carnoy. Brachet-Pappenheim's cytochemical method was employed by introducing some modifications. RNA digestion tests were carried out with ribonuclease extracts prepared from fresh calf pancreas following the method of Kunitz (33) and suggestions by Stowell and Zorzoli (53).

Brachet (6) found the stainability of tissues by methyl green and pyronin variable and their mechanism dependent on many factors. Also in our preparations the usual procedures, as described by Pappenheim (44), Unna (57), Romeis (48), Scudder (49) and Brachet (4, 6) often failed to yield sufficient differentiation of cell elements. Modified procedures suitable for some fixatives were elaborated partially by slightly changing the pH-values of reagents involved. The changes of pH, according to Brachet, do not influence the selectivity of the dye-binding by tissues. Yet, even changes in the capacity of general dyebinding and in colour tints of both stain components can result in a more pronounced distinction of various structures, as shown also by Gerola and Vannini (24). Moreover, the introduction of iso-propyl alcohol into dehydrating baths was useful in securing controllable and effective post-staining differentiation.

To prevent the possible influence of polysaccharides and similar substances present in the pituitary (16, 45), the sections can before the staining be submitted to the oxidizing action of periodic acid, following the methods of McManus (40) or Hotchkiss (29). This treatment does not noticeably affect the nucleic acids and their stainability.

The following procedure was used.

Five slides (a, b, c, d, e) each bearing two adjacent sections of a ribbon in a definite order. Sections of 4 μ, attached on slides with distilled water only.

Oxidation pretreatment. Put slides c, d, e from dist. water into the 0.4–0.6 per cent solution of periodic acid (in dist. w.) for 5–10 min. Wash slides first in dist. w., then in tap water, and again in dist. w., very thoroughly.

Digestion test. Put one (c) of the three (oxidized) slides into ribonuclease sol. at

65° C for 60 min., another (d) into dist. w. at 65° C for 60 min. Rinse the first one in dist. w. thoroughly.

Staining and differentiation. The most suitable mixture for pituitaries fixed according to Carnoy proved to be of the composition: methyl green (Schering's 00, used) 200 mg, pyronin (Grubler's) 250 to 300 mg, ethyl alcohol (propyl alcohol) 2–4 ml, glycerin 12 ml and 0.33 per cent sol. of phenol ad 100 ml. The pH of such a freshly prepared solution, measured with a glass electrode, was ca. 3.85. (The use of stains in buffered acetate and citrate solutions do not give any better results.)

Put slides a, c, d, e from dist. w. into the stain-solution for ca. 5 hours (freshly prepared stain). Rinse and differentiate in dist. w. ca. 3 min. (which chiefly takes away the superfluous methyl green). Dry the slides (but not the sections) with filter paper. Dehydrate and differentiate in a mixture of absolute ethyl alcohol and isopropyl alcohol (100 : 10 to 20). Differentiation time: 15 sec. to some min.; the control under microscope recommended. Continue to dehydrate in absolute ethyl alcohol (2 changes) for 30 sec. Clear it in xylene (2 changes) and mount in neutral Canada balsam.

The duration of staining (especially in ripened stain mixtures) and of the differentiation time (by using various ethyl- and propyl alcohol proportions) can be varied considerably. In very ripe stain mixtures it often suffices only to stain for a few minutes.

Identification of cell types. Stain the fifth slide (b) in one of the common mixtures used in staining the anterior pituitary, introducing a mordant pretreatment when needed.

An optical separation of RNA localizations in preparations was carried out by using narrow cut filters, like Farrand's interference filter of peak wave-length 550 mμ and Ilford's Spectrum filter yellow-green.

RESULTS AND DISCUSSION

The topography of various cell types in the anterior pituitary of the guinea pig is very characteristic and relatively constant (Abolinš, 2). Predominantly acidophilic and basophilic-chromophobe areas, respectively, are recognized in all the sections. The main part of the RNA detected in the anterior pituitary of the guinea pig by means of pyronin is, according to our observations, localized in cells of acidophilic areas. Yet, there are also in the basophilic-chromophobe areas cells present containing RNA (see Fig. 1). A more detailed description of the distribution of RNA upon various cell types will follow in another paper. RNA is always present in the nucleoli, but in varying quantities; moreover also the sizes of the nucleoli vary. The nucleolus-associated chromatin stained by methyl green in bluish can be observed in a number of cells. In the cytoplasm the RNA can frequently be observed in various quantities, partially or completely covering the surface of the nucleus. Less frequently RNA appears in the whole cytoplasm, often unipolarly

localized. Some cells reveal a characteristic condensation of RNA masses at the cell periphery. The substance appears almost always in the form of fibers and networks of variable coarseness. Fig. a–g of Table I show different typical pictures of an RNA localization which on the whole form a closed system of stages always reappearing in similar functional conditions. Fig. a shows a nucleus (among others) with a very great nucleolus-heterochromatin complex (which has optically not been sufficiently isolated on the photograph). On the surface of the nucleus a thin layer of RNA can be observed. To the left a part of this layer has lifted from the surface of the nucleus. In Fig. b the nucleolus-heterochromatin complex became smaller, whereas the RNA layer grew thicker. In both cases, but especially in the last one, the nuclei have extraordinarily great volumes. Both stages appear in young secretory cells with relatively little and apparently feeble differentiated cytoplasmic body. The stages c–f appear in active secretory cells of various advanced degrees of granulation and secretion production respectively. In all the three stages the nucleolus-associated heterochromatin cannot be observed any longer and the nucleoli are relatively small. The main part of RNA is gradually removed from the surface of the nucleus into the cytoplasmic body itself, towards the cell periphery.

In the great exhausted cells with vacuolized cytoplasm (stage h) only the distal cell periphery exhibits sometimes very compact masses of RNA. The complex nucleolus-heterochromatin appears once more. The great "light" degranulated secretory cells (stage g) have, apart from accidentally scarce particles, no RNA at all in the cytoplasm; the nucleolus-heterochromatin complex, however, being extremely great. There is reason to believe that the cell of stage f presents a transition from stages d and e to stages g and h. The great activity of the nucleolus-associated heterochromatin and of RNA of the nucleolus in the last two stages probably indicates the commenced cell restitution act and the inception of a new secretion cycle.

The work by Caspersson and his co-workers on the microspectrophotometry of proteins and nucleotides has produced many arguments for accepting the intimate correlation between the amount of RNA present in the cell and the rate of its protein synthesis. Caspersson (8, 9, 14, 15) concluded that large amounts of protein are synthetized in cells where RNA is abundant in the nucleoli, around the nucleus, or in the cytoplasm. Detailed cytochemical investigations, especially concerning the development of insects and amphibians, led Brachet (4–6) almost simultaneously to analogous conclusions. A number of other investigators (30, 31, 32, 36, 52, 54) have supported the above findings. The role of RNA in the synthesis of protein

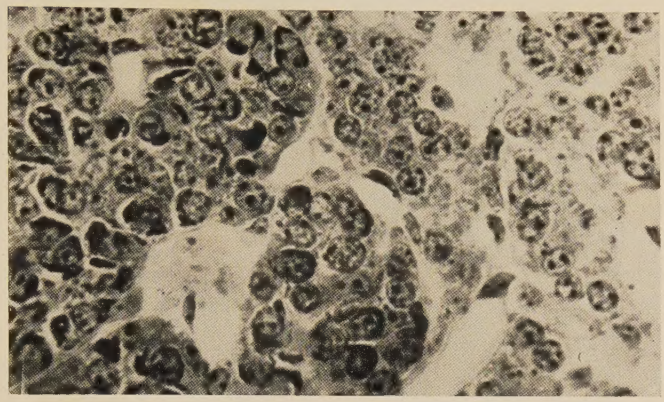


Fig. 1. A part of the frontal section from the medial region of the anterior pituitary of the guinea pig. Dark RNA masses in the nucleoli and the cytoplasm, stained by pyronin, mainly in the predominantly acidophilic area at the left. DNA of the nuclei stained by methyl green. Methyl green-pyronin. Ilford Spectrum yellow-green filter. 500 ×.

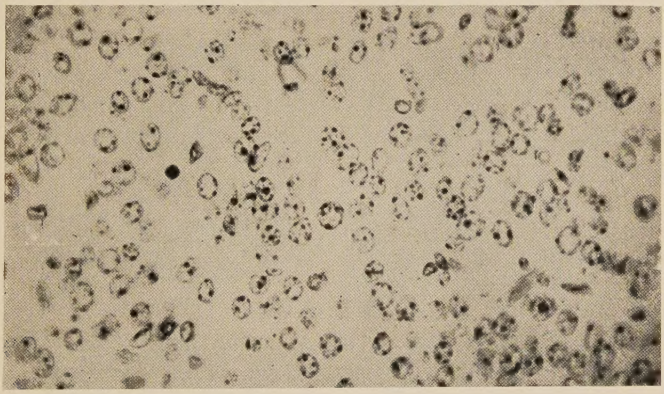


Fig. 2. A part of the anterior pituitary of the guinea pig adequate to that of Fig. 1 (but from a nonadjacent section). Disappearance of RNA in consequence of enzymic digestion. Only DNA present in the nuclei stained by methyl green. Ribonuclease test. Ilford Spectrum yellow-green filter. 500 ×.

is still obscure. Yet, it is clear that the increase of RNA in the cell under definite conditions and the simultaneous enhancement of protein synthesis are not independent parallel proceedings. The increase of the rate of cytoplasmic protein synthesis is initiated by a *precedent* RNA accumulation in the nucleolus and then at the surface of the nucleus, and in the cytoplasm. This has been stated with regard to the pancreas (12), the liver (36, 52), the nerve cell (25, 26, 28, 30, 31), the immature blood stem-cell (54);

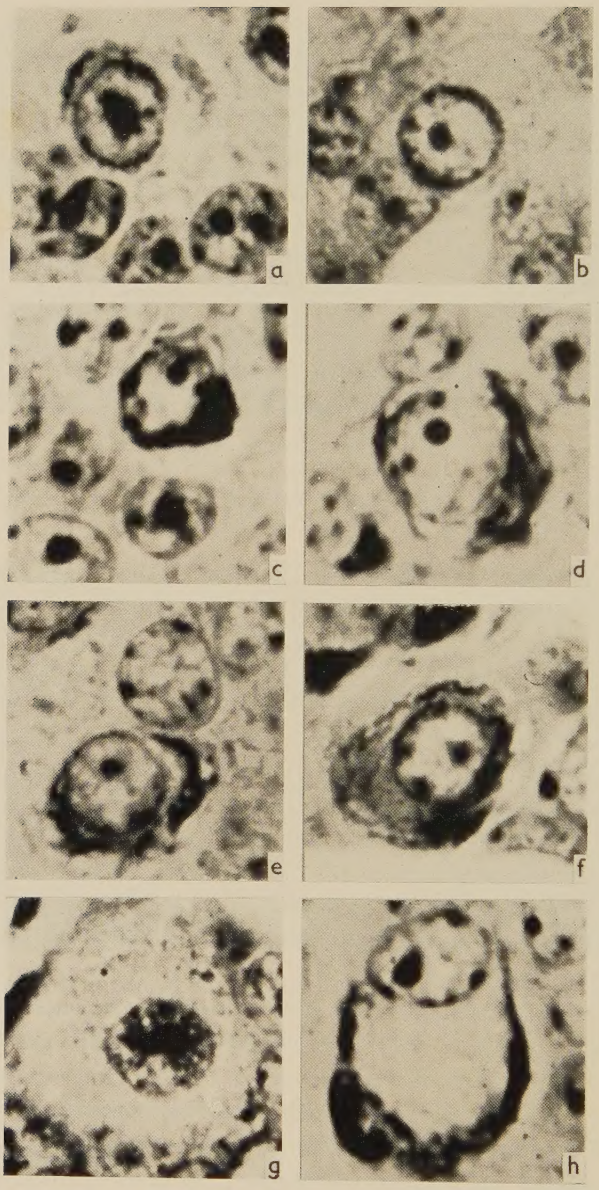


Plate I. Different stages of the RNA localization in secretory cells of the anterior pituitary of the guinea pig. (In the descriptions attention was only paid to the characteristic picture of each figure.)

Fig. a–b. Early secretory stages. RNA present in the nucleoli and on the surface of the nuclei.
Fig. c–e. More advanced stages. Reduction of the volumes of the nucleoli and of nucleoli-associated chromatin. The amount of RNA localized on the surfae of the nuclei increased. RNA appears also at some distance from the nuclei in the cytoplasm.
Fig. f–h. Final stages of the secretory act. f–a cell in the degranulation process with RNA spread over the whole cytoplasm; g–great "light" degranulated cell almost without RNA in the cytoplasm; h–great vacuolized cell with RNA condensed at the cell periphery. Methyl green-pyronin. Farrand's interference filter of peak wave-length 550 m. Photomicrographs 1 800 to 2 400 ×.

the embryos of mice and the liver cells (32). On the basis of these statements Caspersson, in his recent monograph (11), calls the nucleolus-associated chromatin and the RNA complex the cytoplasmic protein-forming system.

Our results reveal the existence of such a system in secretory cells of the anterior pituitary of the guinea pig. The pictures of RNA in different stages described above are closely similar to those discovered in secretory, nerve and blood cells by ultraviolet absorption analysis (12, 26, 28, 30, 31, 54). Though the occurence of RNA in the anterior pituitary has been noted already previously, the present paper gives the first record of the existence of the *entire RNA-system in direct correlation with the secretory function of the anterior pituitary.* To our knowledge, it is also the first report that the cytoplasmic protein-forming system as a whole can be revealed by means of Brachet-Pappenheim's cytochemical method. An approach to the quantitative evaluation of RNA changes (and to the standardization of the methyl green-pyronin cytochemical method) may, probably, be attained by comparing the ultraviolet absorption and the pyronin reaction within the same or closely adjacent sections (of frozen-dried material). It would be so much more important to prove the results gained from the cytochemical method by means of ultraviolet absorption, as the recent findings by Kurnick (34), and Kurnick and Mirsky (35) seeem to show that pyronin stains both RNA and depolymerized DNA with the same colour. It may be added that in the pituitary of man (personal communication by B. Thorell) and that of the rat (pers. commun. by R. Luft) the RNA was already observed by means of Caspersson's method.

As yet we know hardly anything concerning the hormone production mechanism in secretory cells of the anterior pituitary. The work of pituitary chemists (22, 37) revealed that out of six hormones of the anterior pituitary, hitherto isolated, three (growth hormone, prolactin, and ACTH) are pure proteins, whereas the remaining three (FSH, LH, and thyrotrophic hormone) are glycoproteins. In view of these basic facts it was only to be expected that the cytoplasmic protein-forming system, characteristic of other protein-synthetizing cells, should be found also in cells of anterior pituitary. Another question arises as to whether there is any principal difference in protein (mainly in acidophile cells) and glycoprotein (mainly in basophile cells) formation mechanisms. It has been shown (1, 3) that the cytochemical reaction of alkaline phosphatase is much stronger in acidophile cells than in basophile ones. The reaction of the typical acid phosphatase (pH 5.5) is, however, nearly similar in cells of both types.

Modern biochemical research has established a correlation between phosphate turnover, nucleic acids, as well as protein synthesis. Organic phosphate-bonds appear to be the source of energy for synthetic activities (18, 38). According to Spiegelmann (50), it would not appear impossible that, in the synthesis of protein, phosphorylated proteins should have a determining significance. It has been suggested (42, 51) that especially nucleoproteins might be the specific energy donors of protein synthesis.

The comparative cytochemical studies on phosphatases, RNA, and the protein content of acidophile and basophile cells during the secretion cycle, in which we have been engaged for some time, will perhaps offer the opportunity to correlate the cytological findings in the pituitary with the biochemical points of view mentioned above more in detail. As yet, the typical pictures of the RNA localization in the anterior pituitary of the guinea pig, may, on the basis of the general conception of the RNA function, be considered only as indicators of various stages of protein production in secretory cells.

SUMMARY

The Brachet-Pappenheim cytochemical RNA-detection method was used to reveal the localization of RNA in nuclear and cytoplasmic structures of secretory cells of the anterior pituitary in various functional states. The methyl green-pyronin staining standard procedure was slightly modified by changing the acidity of the stain mixture, prolonging the staining and differentiation time, and introducing iso-propyl alcohol in the differentiation.

The main part of the RNA detected by means of pyronin was found to be distributed upon the predominantly acidophilic area. The pictures of RNA-localization, obtained in early, more advanced and final stages of the secretory act, were closely similar to those discovered in secretory cells of other glands, and in nerve cells, by ultraviolet absorption analysis. It is the first time properly with regard to the pituitary, that a similar, cytoplasmic protein synthesis indicating RNA-system, *as a whole*, could be revealed by means of Brachet-Pappenheim's cytochemical method. Relation of the RNA as to the various cell types in the pituitary, and to the appearance of phosphomonoesterases is discussed.

REFERENCES

1. Abolinš, L., *Nature*, **161**, 556 (1948).
2. —— *Arkiv Zool.*, **41 A**, No 17 (1948).
3. Abolinš, L., and Abolinš, A., *Nature*, **164**, 455 (1949).
4. Brachet, J., *Compt. Rend. Soc. Biol.*, **133**, 88 (1940).
5. —— *Enzymologia*, **10**, 87 (1941).
6. Brachet, J., *Arch. Biol.*, **53**, 207 (1942).
7. Caspersson, T., *Skand. Arch. Physiol.*, **73**, Suppl. 8 (1936).

8. —— *Arch. Exptl. Zellforsch. Gewebezücht.*, **22**, 655 (1939).
9. —— *Naturwissenschaften*, **29**, 33 (1941).
10. —— *Symposia Soc. Exptl. Biol.*, **1**, 127 (1947).
11. —— Cell growth and cell function. W. W. Norton Co., New York, 1950.
12. Caspersson, T., Landström-Hydén, H., and Aquilonius, L., *Chromosoma*, **2**, 111 (1941).
13. Caspersson, T., and Santesson, L., *Acta Radiol., Suppl.* **46** (1942).
14. Caspersson, T., and Schultz, J., *Nature*, **143**, 602 (1939).
15. —— *Proc. Natl. Acad. Sci.*, **26**, 507 (1940).
16. Catchpole, H. R., *J. Endocrinol.*, **6**, 218 (1949).
17. Celestino da Costa, A., *Compt. Rend. Assoc. Anat.*, **18**, 107 (1923).
18. Cori, G. T., Swanson, M. A., and Cori, C. F., *Federation Proc.*, **4**, 234 (1945).
19. Dempsey, E. W., and Wislocki, G. B., *Amer. J. Anat.*, **76**, 277 (1945).
20. Desclaux, P., *Arch. Anat. Microscop. et Morphol. Exptl.*, **36–37**, 91 (1946–47).
21. Desclin, L., *Compt. Rend. Soc. Biol.*, **133**, 457 (1940).
22. van Dyke, H. B., Chow, B. F., Fevold, H. L., Long, C. N., White, A. *et al.*, *Ann. N. Y. Acad. Sci.*, **43**, art. 6 (1943).
23. Feulgen, R., and Rossenbeck, H., *Hoppe-Seylers Z. physiol. Chemie*, **135**, 203 (1924).
24. Gerola, F. M., and Vannini, E., *Atti Acad. Naz. Lincei*, **5**, 77 (1948).
25. Gersh, I., and Bodian, D., *Biol. Symposia*, **10**, 163 (1943).
26. Hamberger, C. A., and Hydén, H., *Acta Oto-Laryngol.*, **75**, 53 (1949).
27. Herlant, M., *Arch. Biol.*, **54**, 225 (1943).
28. Hochberg, J., and Hydén, H., *Acta Physiol. Scand.*, **17**, Suppl. 60 (1949).
29. Hotchkiss, R. D., *Arch. Biochem.*, **16**, 131 (1948).
30. Hydén, H., *Acta Physiol. Scand.*, **6**, Suppl. 17 (1943).
31. —— *Z. mikroskop. anat. Forsch.*, **54**, 96 (1943).
32. Jeener, R., and Szafarz, D., *Arch. Biochem.*, **26**, 54 (1950).
33. Kunitz, M., *J. Gen. Physiol.*, **24**, 15 (1940).
34. Kurnick, N. B., *ibid.*, **33**, 243 (1950).
35. Kurnick, N. B., and Mirsky, A. E., *ibid.*, **33**, 265 (1950).
36. Lagerstedt, S., *Acta Anat.*, Suppl. 9 (1949).
37. Li, C. H., and Evans, H. M., The Hormones, Ed. by G. Pincus, Academic Press, New York, **1**, p. 631, 1948.
38. Lipmann, F., *Advances in Enzymol.*, **1**, 99 (1941).
39. Lison, L., Abstr. Comm. 1. Intern. Congr. Bioch., 266, 1949.
40. McManus, I. F., *Nature*, **158**, 202 (1946).
41. Michaelis, L., *Cold Spring Harbor Symposia Quant. Biol.*, **12**, 131 (1947).
42. Muller, H. I., *Proc. Roy. Soc.*, **134**, **1** (1947).
43. Opie, E. L., and Lavin, G. L., *J. Exptl. Med.*, **84**, 107 (1946).
44. Pappenheim, A., *Folia haematol.*, **6**, 51 (1908).
45. Pearse, A. E., *Nature*, **162**, 651 (1948).
46. Pollister, A. W., and Leuchtenberger, C., *Proc. Natl. Acad. Sci.*, **35**, 111 (1949).
47. Racadot, J., Cytogenèse de la glande pituitaire, Nancy, 1949.
48. Romeis, B., Mikroskopische Technik, 15. Aufl., Leibniz Verlag, München, 1948.
49. Scudder, S. A., *Stain Technol.*, **19**, 39 (1944).
50. Spiegelmann, S., *Symposia Soc. Exptl. Biol.*, **2**, 286 (1948).
51. Spiegelmann, S., and Kamen, M., *Science*, **104**, 581 (1946).
52. Stowell, R. E., *Arch. Path.*, **46**, 164 (1948).
53. Stowell, R. E., and Zorzoli, A., *Stain Technol.*, **22**, 51 (1947).
54. Thorell, B., Studies on the formation of cellular substances during blood cell formation, London, 1947.
55. —— *Acta Path.*, **25**, 54 (1948).
56. Tuchmann-Duplessis, H., and Labat, J., *Compt. Rend. Soc. Biol.*, **140**, 240 (1946).
57. Unna, P. G., *Monatschr. prakt. Dermatol.*, **35**, 76 (1902).

[1]) Since this paper was submitted, further two noteworthy publications by E. B. Taft (Exptl. Cell Res., **2**, 312 (1951); Stain Technol., **26**, 205 (1951) concerning the specificity of the methyl greenpyronin stain have appeared.

A CYTOPLASMIC FRACTION ACTING ON THE SURFACE LAYERS OF THE *ARBACIA PUNCTULATA* EGGS

ALBERTO MONROY[1] and JOHN RUNNSTRÖM[1]

Marine Biological Laboratory, Woods Hole, Mass.

Received March 15, 1951

According to Runnström (8) and Tyler (11) extracts from frozen and thawed eggs of *Arbacia punctulata* contain some agent which precipitates the jelly coat of intact eggs. This was confirmed by Runnström and Monroy (9) for *Arbacia lixula* from Naples. They found moreover that extracts in KCl of frozen and dried eggs of this latter species did not cause any precipitation of the jelly coat, unless the electrolyte content of the sea water was decreased by addition of distilled water or isotonic cane sugar solution. Crystalline lysozyme acted in the same way as the egg extract. Eggs pretreated with this substance in sea water exhibited a strong precipitation of the jelly upon transfer into diluted sea water or cane sugar solution. Thrombin is also able to precipitate the jelly coat in a medium of low salinity, but not in sea water (6).

The eggs of *Arbacia lixula* sometimes fail to form an elevated normal fertilization membrane upon fertilization. Instead a tight gelatinous rim appears on the surface of the egg. This occurs particularly if the eggs have been kept in sea water for some hours (10). If the eggs were inseminated in presence of egg extract in sea water a normal fertilization membrane was obtained. The presence of the egg extracts brings about an elevation and a hardening of the membrane. Attempts at the isolation of the active fraction of the egg extract were unsuccessful. Monroy and Runnström (6) therefore, took up the problem again working in Woods Hole with *Arbacia punctulata*. The present paper is a fuller report of the results obtained. The work is at present carried further at the Zoological Station in Naples. Motomura (7) has also described a factor which toughens the membrane. He obtained the toughening factor by extracting unfertilized eggs of *Strongylocentrotus pulcherrimus* or recently fertilized eggs of *Temnopleurus hardwickii* and *Clypeaster japonicus* with acidified sea water. The extracted eggs lose their capac-

[1] Present addresses: Ist. Anat. Comp., Università Palermo, Italy, and Wenner-Gren Institute, University of Stockholm, Sweden, respectively.

ity of forming a tough membrane, but this capacity is restored when the extract neutralized to the pH of the sea water is added to the egg suspension. These results recall very much those obtained on *Arbacia lixula* (9, 10). Our colleague, G. Kriszat, has fully confirmed Motomura's results for the eggs of *Psammechinus miliaris* from the Swedish West Coast.

The isolation of a cytoplasmic fraction containing the egg antifertilizin. Extracts in 1 M KCl of frozen dried eggs of *Arbacia punctulata*, dialysed against sea water, cause a strong and immediate precipitation of the jelly coat of intact eggs of the same species in sea water. This seems to indicate that the factor responsible for this reaction is more soluble in the eggs of *A. punctulata* than in those of *A. lixula*. Probably the structural elements to which it is bound are more easily disrupted and extracted in *A. punctulata* than in *A. lixula* (see below). As a matter of fact one of the writers (A.M.) has found that the N extractable with 1 M KCl from frozen dried eggs of *Arbacia lixula* from Naples amounts to about 65 per cent of the total N content of the egg, whereas in the case of the eggs of *Arbacia punctulata* from Woods Hole this value reaches 77–80 per cent.

The factor which is obtained by KCl-extraction of the frozen and dried eggs of *Arbacia lixula* eggs may be a less active breakdown product of the complete agent. The combination between jelly substance and the supposed breakdown product is more easily dissociable by electrolytes than the combination jelly substance + the complete agent. This latter agent is present also in the *Arbacia lixula* eggs as follows from the fact, see above, that extracts from frozen and thawed eggs precipitate the jelly even at the salinity of sea water.

An attempt was made at the isolation of the complete agent. Frozen dried eggs of *Arbacia punctulata* (deprived of the jelly coat by treatment with acid sea water) were extracted in 1 M KCl and the insoluble parts sedimented by centrifugation in the cold at 6 000 × g for 1 hour. The clear extract was then submitted to two high speed centrifugations in the Spinco preparative ultracentrifuge. The progress of the fractionation was followed electrophoretically (in the Perkin-Elmer electrophoretic apparatus) after dialysis of the fractions against 0.1 N $NaHCO_3$, pH = 8.6, ionic strength 0.1. The jelly coat precipitating activity was tested after dialysis of the extracts against sea water. From a suspension of eggs washed several times, aliquots were pipetted out with a graduated pipette. To this the solution to be tested was added and the proportions of the mixture were adjusted so as to have a final volume of 2 cc. The concentration of the tested solutions was determined by N determination.

As shown in the diagram of fig. 1, a first centrifugation at 25 000 × g for 45 min. results in the sedimentation of a fraction that forms a pellet at the bottom of the tube and can be redissolved incompletely and with great difficulty. This solution proved to be wholly deprived of jelly coat precipitating activity. In consideration

of the difficulty of bringing this fraction into solution the objection may be raised, however, that higher concentrations of the fraction would have caused jelly precipitation. The final concentration of the tested solution was 0.152 mg N/ml. In more cautious formulation it was found that the fraction was still inactive at this concentration. The activity if any must thus be low.

The supernatant of the first centrifugation was then submitted to a second ultracentrifugation at 155 000 × g for two hours. Two layers thus separated: one heavily pigmented bottom layer of gelatinous appearence and an upper layer slightly yellow in colour. The upper layer was pipetted off and the sedimented layer resuspended in buffer and submitted to a second ultracentrifugation at the same speed for 2 hours. The sedimented fraction was then redissolved in buffer. Sediment and supernatant were then checked for their jelly coat precipitating ability (after dialysis against sea water) and examined electrophoretically (after dialysis against bicarbonate buffer). The jelly coat precipitating activity was found then to be entirely confined to the pigmented sedimented fraction, the supernatant being completely devoid of effect. The jelly coat precipitating effect could be detected in the solution of the sediment until a final concentration in the mixture of 0.08 mg N, while the supernatant was still inactive at a final concentration of 0.52 mg N.

The active fractions could be obtained from unfertilized and fertilized eggs as well. No differences could be observed with respect to activity.

The electrophoretic analysis shows that the active fraction contains two distinct components having a mobility of —4.0 and —5.1 × 10^5 respectively. The pigment seems to migrate with both components. An attempt at the separation of the two components has failed. A more detailed analysis of the electrophoretic patterns of the different fractions obtained by ultracentrifugation of the extracts will be given elsewhere. However, the slower component of the active fraction can be identified by its mobility with the largest slow component of the total extract while the identification of the faster one is uncertain. Analysed in the analytical ultracentrifuge[1] the active fraction showed two components (fig. 2) rather close to each other. The main fast component is associated with the entire pigment. This indicates that further attempts at the purification of the active fraction are needed in order to decide definitely about the role of the pigment in the activity.

In one experiment, one part of the sedimented active fraction was treated with a dilute solution of crystalline pepsine in HCl at pH 1.4 at the room temperature for 4 hours. The mixture was then diluted 5 times with sea water and pH brought to 7.7 by adding a solid mixture of $NaHCO_3$–Na_2CO_3. The solution proved to have completely lost its activity.

Both the active fraction and the inactive supernatant show a slight absorption in the U.V. at 2 600 Å. This absorption is somehwat higher in the supernatant. That seems to rule out the possibility that nucleic acid has any part in the precipitation reaction.

---

[1] We are indebted for this analysis to Dr. J. C. Bugher and Mr. Baker of the International Health Division at the Rockefeller Institute.

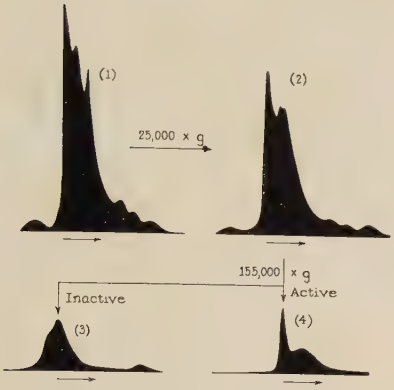


Fig. 1.

Fig. 1. Electrophoretic patterns of a total KCl extract (1) of unfertilized eggs of *Arbacia punctulata* and of the fractions (2–4) obtained by ultracentrifugation. Electrophoresis in 0.1 N $NaHCO_3$, ionic strength 0.1, pH 8.6, Pot.grad. 6.2 Volts/cm. Patterns drawn after photographs taken after 90 min:s run.

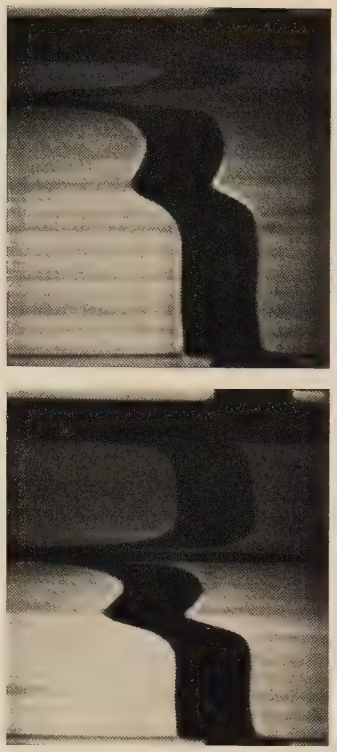


Fig. 2. Sedimentation in the ultracentrifuge of the active fraction of an extract of unfertilized eggs of *Arbacia punctulata*.

Fig. 2.

If the active fraction acted upon jelly free eggs, the membrane of the unfertilized egg often seemed more refractile. The effect of the agent does thus not seem to be directed only on the jelly coat, but also on the underlying layers of the egg surface.

The active fraction corresponds to the egg antifertilizin, the presence of which was postulated already by Lillie (5) on the basis of rather crude experiments. According to definition antifertilizin inhibits the agglutination action of the fertilizin, corresponding to or contained in the jelly coat substance brought into solution. Table I shows one experiment which proves that our fraction possesses this antifertilizin action.

It follows further that both unfertilized and fertilized eggs contain antifertilizin. This agrees with the fact reported above that the jelly coat is precipitated by extracts both from unfertilized and fertilized eggs.

Lillie assumed that the antifertilizin is bound upon the fertilization of the

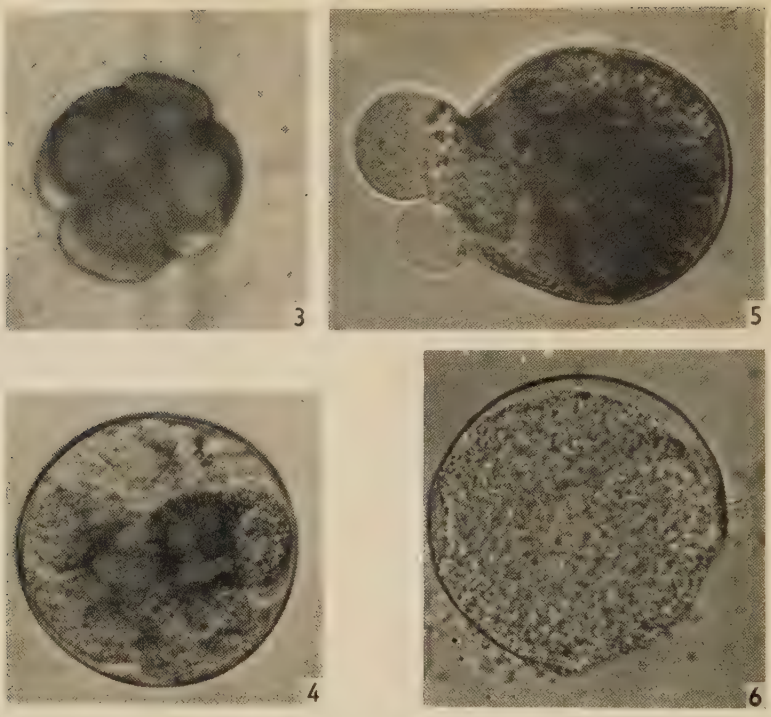


Fig. 3. Control egg of *Arbacia punctulata* with membrane of normal thickness.
Fig. 4. Egg of *Arbacia punctulata*, fertilized in presence of iodosobenzoic acid. Thick, tough membrane around the egg which (140 minutes after fertilization) has undergone cytolysis.
Fig. 5. Egg of *Arbacia punctulata* submitted to centrifugation before fertilization, and afterwards fertilized in presence of iodosobenzoic acid. Photographed about 150 minutes after fertilization.
Fig. 6. Unfertilized eggs of *Psammechinus miliaris* devoid of jelly coat kept in sea water for about 20 hours. Separation of a finely granular layer and formation of a birefringent membrane above this layer.

egg. This seems unlikely in view of our experiments which, however, have to be considerably more varied and extended.

The antifertilizin as a factor hardening the fertilization membrane. Eggs of *Arbacia punctulata* were fertilized in the presence of iodosobenzoic acid (IBA); 0.00264 M was found to be an appropriate concentration for our purpose. Table II gives some details from one experiment; see also figs. 3 and 4.

The fertilization membrane formed in presence of the IBA is considerably thicker and more refractile than that appearing in the control. These changes could also be demonstrated in the polarization microscope. The thickened

TABLE I

Agglutination of spermatozoa by egg water and its inhibition on addition of pigmented egg fractions. The fraction from unfertilized eggs contained 1.44 mg N/ml, that from fertilized eggs 1.57 mg N/ml.

The following mixtures were prepared:

a) 1 ml egg water + 2 ml pure sea water.

b) 1 ml egg water + 2 ml antifertilizin from unfertilized eggs.

c) 1 ml egg water + 2 ml antifertilizin from fertilized eggs.

	Agglutination
1 dr. of a) + 1 dr. spermsuspension	+ +
1 dr. of b) + 1 dr. spermsuspension	——
1 dr. of c) + 1 dr. spermsuspension	——

TABLE II

Unfertilized eggs were pretreated for 3 hours 40 minutes in 0.0026 M iodosobenzoic acid (IBA). As control served eggs in pure sea water. The samples were inseminated simultaneously.

Time in minutes after insemination	Control	IBA
2	100 per cent formation of a fertilization membrane	100 per cent formation of a fertilization membrane which is thicker and more refractile than that of the control.
80	2 and 4 cell stages, membrane unchanged	2-cell stages, the membrane is in most cases considerably more conspicuous than in the control.
140	Cleavage has continued normally	In 23 per cent of the eggs cleavage delayed in comparison with the control. In 77 per cent cytolysis with escape of pigment. Membrane considerably thicker in the cytolyzed than in the cleaved egg, cf. figs. 3 and 4.

membrane gave a much more distinct cross in the extinction positions and was much brighter in the intermediate positions than the membrane of the control. This indicates a definite increase in the retardation values. We had no opportunity of making quantitative estimations of these.

The thickening of the membrane was correlated with the escape of pigment. This was more easily detectable after some hours, but the escape of pigment was no doubt responsible for the membrane thickening already from the beginning. The thickening may take place also in rather late stages of cleavage, depending on the stage in which the eggs are so affected that a strong leakage of pigment occurs.

A precipitation of the jelly coat may sometimes also occur following fertilization in presence of IBA. This precipitation is evidently conditioned by a further diffusion of the pigment through the fertilization membrane. The precipitation is of a granular character, with no sharp exterior borderline, in contradistinction to the precipitation with sharp borderline which is formed when the antifertilizin is added from outside the egg.

Even if the eggs cytolyze within a thickened membrane, this latter may break up at a certain weak point. Eggs raised in presence of for example 0.001 M IBA developed until the blastula stage, but at the time of hatching the dissolution of the membrane was strongly inhibited. Some larvae may escape, but this takes place without a breakdown of the membrane. The larvae force themselves through a narrow opening in the membrane and this remains empty on the bottom of the dish.

The conclusive proof of the causal relation between pigment escape and thickening of the membrane was obtained in experiments with unfertilized eggs which had been submitted to centrifuging. In this way the pigment was packed at the centrifugal egg pole. Eggs pretreated in this way were transferred to 0.0026 M IBA in sea water and here inseminated after exposure for 5–10 minutes to the IBA. Both non centrifuged and centrifuged but not IBA-treated eggs were kept as controls. They were fertilized at the same time as the centrifuged eggs subjected to IBA. Ten to fifteen minutes after insemination, these latter eggs showed a conspicuous thickening of the membrane, but this was limited to the pigmented egg pole, where the escape of pigment occurred. The opposite side of the membrane was extremely thin. When the eggs cytolyzed subsequent to a prolonged treatment with IBA the membrane at the non pigmented side made practically no resistance to the swelling blisters, see fig. 5. On the opposite side the thickened membrane is visible; the increased retardation of this part of the membrane as compared with the control was easily demonstrable with the polarization microscope.

In conjunction with the elevation of the fertilization membrane a contraction of the egg surface occurs. This contraction has been subject to a cinematographic study by W. and G. Kuhl (4). It was observed repeatedly that the contraction was mainly confined to the pigmented egg pole of the centrifuged eggs. This suggests that also the contraction is due to the release of substances from the particulate matter concentrated at the centrifugal egg pole. Even without addition of IBA the difference between centripetal and centrifugal part of the egg is obvious. In the former the membrane is much thinner than in the latter (2, 1). The action of IBA, however, accentuates the difference.

The particulate localization of antifertilizin in eggs of Psammechinus miliaris. A fortuitous observation gave supplementary information about the localization and role of the antifertilizin in eggs of *Psammechinus miliaris*. By brief exposure (for 30 seconds) to acid sea water (pH around 4) most of the eggs were deprived of their jelly coats. The eggs were then kept for 20 hours in normal sea water. During the course of this time a granular material was separated from the fibrous cytoplasm. The granules were of uniform size and were probably embedded in a hyaline ground substance. They were rather pale having given off their pigment. This occurs always when the granules are exposed to a Ca containing medium, see Hultin (3). The granules formed a layer all around the egg or the granular layer was crescentlike, covering only one part of the bulk of cytoplasm, see fig. 6.

The granular region was covered by a refractile membrane. In the eggs with crescentlike granular layer the refractile membrane was never seen to extend beyond the granular region. The membrane was birefringent, the value of retardation being only 20 -30 per cent lower than that found in freshly elevated normal fertilization membranes. There is thus no doubt that the refractile membrane corresponds to a fertilization membrane. Its strict congruity with the granular region observed in several hundred eggs in repeated experiments prove the causal relation between the separation of granules and the appearance of the refractile membrane.

In a minority of eggs the jelly coat had not been removed by the treatment. It was precipitated and exhibited a birefringence, negative with respect to the radial direction as in the membrane. The same coincidence between effect on membrane and on jelly coat was thus found here as in the cases reported above.

DISCUSSION

The above data bring a first approach towards the isolation of a factor which precipitates the jelly coat. To this factor the designation "antifertilizin" (Lillie) should be applied. It inhibits the agglutinating action of the egg water. It must be inferred, however, that the same factor or at least a factor belonging to the same fraction has a definite hardening influence on the fertilization membrane. The very often observed parallel action on the jelly coat and on the membrane speaks in favor of a unitary conception. It seems rather probable that antifertilizin is secreted upon fertilization in conjunction with the slight contraction of the egg surface which accompanies the membrane elevation. The membrane hardening substance may be obtained in a more incomplete form, in which it is unable to precipitate the jelly coat in presence of the electrolytes of the sea water. Only after dilution of the medium a precipitation is brought about. This "incomplete" factor may correspond to a somewhat degraded antifertilizin. Motomura's membrane toughening factor does not seem to precipitate the jelly coat in sea water medium and is therefore perhaps "incomplete" in the above sense.

The antifertilizin is included in certain particles—the pigment granules—

which perhaps may be regarded as modified mitochondria. From a general point of view it is interesting that a physiologically highly active substance like the antifertilizin is released from particulate components under the action of a stimulus which spreads from the point of attachment of the spermatozoon.

SUMMARY

A jelly precipitating factor was obtained from the eggs of *Arbacia punctulata*. The eggs were frozen and dried, extracted with 1 M KCl solution and thereafter submitted to ultracentrifugation. A fraction sedimenting following centrifugation for 45 minutes at 25 000 × g presented no demonstrable activity. A second fraction, however, obtained following centrifugation for two hours at 155 000 × g gave a highly active fraction, whereas the supernatant proved to be inactive. The active fraction was shown by electrophoresis and ultracentrifugation to contain at least two components.

The jelly precipitating factor inhibits the agglutination of the spermatozoa by the fertilizin and is thus identical with the egg antifertilizin.

In eggs of *Arbacia punctulata* a factor was demonstrated which changes the membrane so as to render this tougher and more refractile. This factor is probably identical with antifertilizin or at least closely adhering to this. The factor was shown to escape from the pigment vacuoles of the egg.

Supplementary observations indicate that also in eggs of *Psammechinus miliaris* substances given off from particulate components precipitate the jelly and toughen the membrane as well.

The writers are very much indebted to the authorities of the Marine Biological Laboratory of Woods Hole for generous help and excellent working facilities. The work was supported by the Rockefeller Foundation and the Swedish Natural Science Research Council.

REFERENCES

1. Costello, D. P., *Physiol. Zool.*, **8**, 65 (1935).
2. Harvey, E. B., Personal communication.
3. Hultin, T., *Exptl. Cell Res.*, **1**, 272 (1950).
4. Kuhl, G., and Kuhl, W., *Zool. Jahrb.*, **70**, 1 (1949).
5. Lillie, F. R., Problems of Fertilization, Chicago University Press, 1919.
6. Monroy, A., and Runnström, J., *Biol. Bull.*, **99**, 339 (1950).
7. Motomura, I., *Sci. Repts. Tôhoku Univ.*, **18**, 561 (1950).
8. Runnström, J., *Biol. Bull.*, **68**, 327 (1935).
9. Runnström, J., and Monroy, A., *Arkiv Kemi* **2**, 405 (1950).
10. Runnström, J., and Wicklund, E., *Arkiv Zool.*, **1**, 179 (1950).
11. Tyler, A., *Proc. Natl. Acad. Sci.*, **25**, 317 (1939).

EFFECT OF DIRECT X-IRRADIATION ON MAMMALIAN TESTICLES

L. C. FOGG[1] and R. F. COWING[2]

Department of Public Health, Pondville Hospital, Mass.

Received March 21, 1951

The purpose of this investigation is to obtain quantitative data on the biological effects of direct x-irradiation on mammalian testicles as a contribution to fundamental problems of growth, regeneration and differential response of various cellular elements of the testes. The data make possible a comparison of effects of varied dosage in single strains of mice and of rats and a contrast of tissue response at the same dosage. The method of quantitative random sampling indicates a predictable and reproducible series of events some of which are regardless of dosage and others of which are dependent on dosage. It is indicated that at high doses the rats and the mice of the strains used, react in a similar manner.

It has been previously reported (4, 5) that after direct, single exposure, using small doses of 300 r as well as large doses of 1 440 r and 5 050 r, spermatogonia disappear first, followed progressively by the spermatocytes, the spermatids and sperm. The slope of the curve representing decrease in frequency for any germinal element was the same, also no pathologic changes were noted in cells or tissues other than those of the germinal series.

In recent publications having to do with changes produced in mammalian testicles by x-irradiation, Eschenbrenner and Miller (2), Warren (9), Lea (7), and Bloom (1) generally agree that spermatogonia are the most sensitive and that the interstitial cells are relatively radio-resistant when compared to germ cells. Eschenbrenner and Miller (2) utilized a series of doses (50 to 400 r), over-all radiation, to study the damage and revovery pattern following acute roentgen irradiation. They emphasize "that because of its sensitivity and the reproducibility of results, the testis is almost without parallel as a mammalian test organ for measuring the biologic effect of ionizing radiations. It will no doubt prove to be a valuable tool for subsequent studies on the relation between biologic and physical dose of ionizing radia-

[1] Chief of Laboratory, Pondville Hospital, Instructor in Pathology, Harvard University.
[2] Medical Nuclear Physicist.

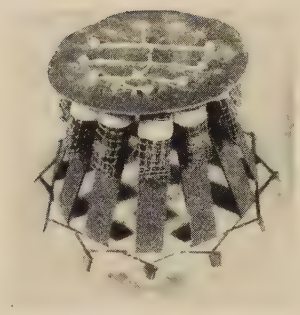


Fig. 1. Shows the method by which the animals were immobilized. The testes appear through the portals of the lead shield.

tions." In their work they reach the tentative conclusion "that in the case of spermatogonia the ionizing radiation causes a graded inhibition of mitoses in proportion to the dose administered. In other words, we probably are not dealing with an all or none effect." They also note, "the pattern of loss and recovery of weights of the testes of mice exposed to 10 mev roentgen rays is the same as that following exposure to 186 kv roentgen rays." There is an increasing body of evidence, therefore, that indicates the biologic response to ionizing radiations follows a pattern. There is, therefore, a need for the accumulation of more quantitative data on the pattern of response. Also, the question arises—is the pattern of response the same when different species are considered? The data presented here contribute to the solution of these problems.

There is general agreement that cells in mitosis, especially the late prophase, are the most sensitive to radiation. There is, however, a lack of experimental data on comparative sensitivity of resting cells, under the same physical conditions of radiation. Those of particular interest are the embryonal type, such as the spermatogonia contrasted with the meiotic cells. Bloom (1), for example, does not agree that mitosis is a predisposing factor toward radiation damage in the testis. Eschenbrenner, Miller and Lorenz (3) have observed that there is a difference between those spermatogonia that divide into other spermatogonia, and the secondary spermatocytes that divide into spermatids as indicated by the sensitivity, although this difference is not evident morphologically.

METHODS

The testes of pure line young adult C 57 black mice, 18 to 25 gm, were subjected to a single exposure of 138 r and in another series animals were subjected to 2 000 r. Studies on doses of 300 r, 1 440 r and 5 050 r have been previously reported (4, 5). Young adult white rats 215 to 285 gms of the Sloniker strain procured originally

for the laboratory of Dr. Warren, Deaconess Hospital, Boston, Massachusetts, and inbred in our laboratory, were similarly treated with doses of 300 r and 4 500 r. These dosages were arbitrarily selected in line with the general belief that rats are more radioresistant than mice. Only the testes were radiated, the body of the animal being protected by a lead shield. The manner in which the animals were immobilized and the method of exposing the testicles have been described (4). The apparatus used for exposing the testicles of the mice is illustrated in fig. 1. The procedure for rats was the same. Animals similarly immobilized but not radiated were used as controls.

Radiation was obtained from a G.E. Maximar, 250 KvP X-Ray Unit and the following factors were used: 100 KvP, 15 Ma Hvl 2.6 mm Al, 187 r per minute at TSD of 20 cm.

For each of the doses indicated, a sufficient number of rats or mice were radiated so that at least four animals could be killed in 2 hours and on the following days post radiation: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 18, 21, 28, 35 and 42. Final observations, not indicated on the figures, were made at 8 and 12 weeks post radiation. Other treated animals were kept for observation and for breeding.

For this study the testes were fixed in Zenker's solution and representative serial sections stained with hematoxylin and eosin.

The frequency of appearance of germ cells in cross sections representative of the whole of both testes was noted by our method of random sampling (4). At a magnification of 430 x, 100 cross sections were examined and the presence of each of the four germinal elements in each was recorded. In a normal testis, an average count reveals that 77 of 100 cross sections contain spermatogonia. The average count in these experiments represents the average from four animals studied for each interval recorded. Additional cytological and histological features were noted such as the Sertoli cells, the interstitial cells, the connective tissue, the lining of the tubules, the blood vessels and the relative size of the tubules.

OBSERVATIONS

The results obtained from direct single-exposure doses of 138 r and 2 000 r on mice will be reported first, then the results on doses of 300 r and 4 500 r on rats followed by a comparison of the two species. Reference to previous reports on the effects of 300 r, 1 440 r and 5 050 r on mice will be made since these contribute to the understanding of changes in response to increased dosage.

Fig. 2 is an example of how the frequency of any germinal element based on our method of random sampling is graphically represented. The observation of the number of times any germinal element, in this case the spermatogonia, appears in 100 cross sections of tubules, chosen at random and representative of the whole of both testes, is the abscissa, while the successive periods post radiation is the ordinate. In all the studies post radiation, there have been evidences of biological variation. This is expressed in the

Fig. 2.

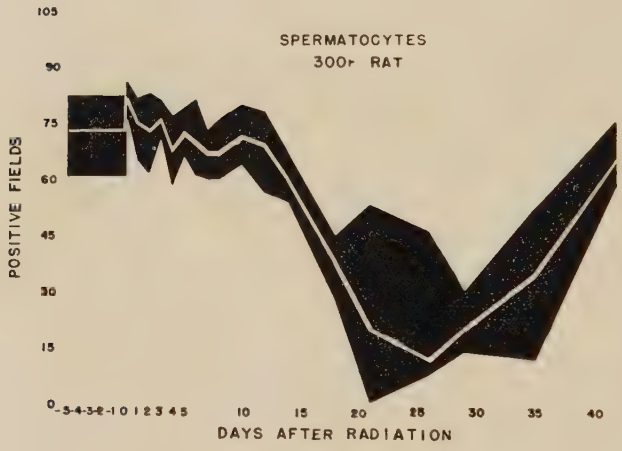


Fig. 3.

Figs. 2–5. Incidence of frequency of each type male germ cell of rat at intervals following direct irradiation, 300 r.

graphs by a black area. The white line represents the average incidence of frequency, and the black represents the extent of variation for each interval. The counts of 10 immobilized but nonradiated animals appear at the left of every graph as a control (—5, —4, —3, —2, —1). Zero days after radiation represents an interval of not more than 2 hours which is the elapsed period from the act of radiation until the tissue is put into fixative. A critical analysis

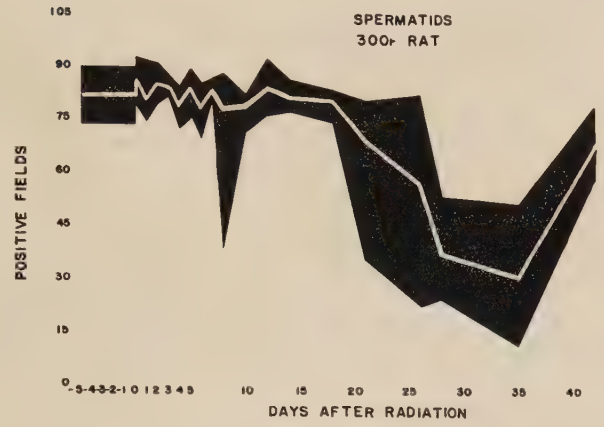


Fig. 4.

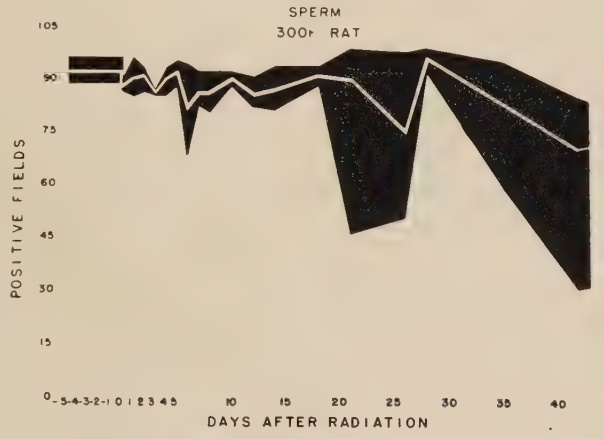


Fig. 5.

of similar graphs has been reported from data obtained on mice after doses of direct irradiation on testes at 300 r, 1 440 r and 5 050 r (4, 5).

In order to evaluate physical radiation dose versus biologic effect, graded doses of 138 r to 5 050 r can now be presented. As several factors are to be considered, certain features will be emphasized, namely 1) control; 2) time when radiation effect is first indicated as lowered incidence of any germinal element; 3) the rate of lowered incidence; 4) the low point or that point of lowest frequency; 5) onset of recovery and 6) the rate and extent of recovery.

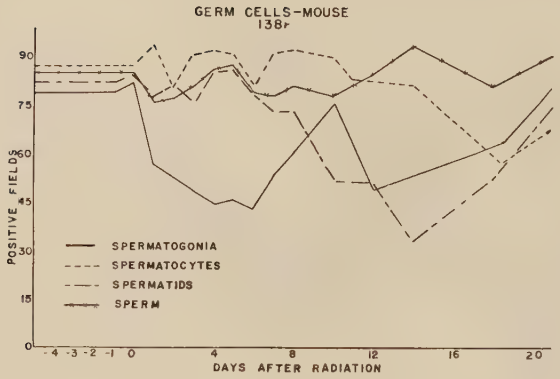


Fig. 6. Incidence of frequency of each type male germ cell at intervals following direct irradiation, 138 r.

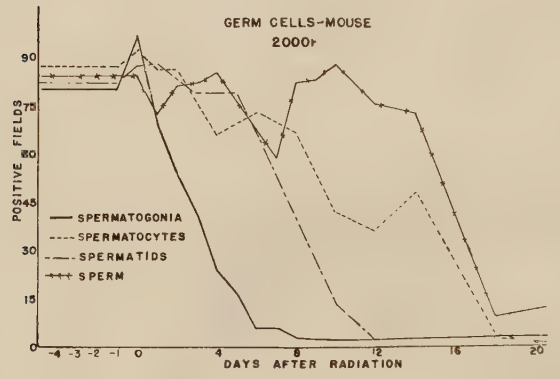


Fig. 7. Incidence of frequency of each type male germ cell at intervals following direct irradiation, 2 000 r.

A comparison of figs. 6 and 7 in relation to the six features listed above should be made. Each graph represents data from a minimum of 80 radiated animals. These graphs should be supplemented by reference to those already published on doses of 300 r, 1 440 r and 5 050 r. This represents a series which at the low dose reveals no permanent damage and at the high dose causes total and permanent loss of the germ cells. This new data must necessarily be considered in its relation to the other dosages of the series. In the control animals there is some variation but of the 10 animals counted it appeared, on the average, that 77 of 100 cross sections of tubules examined showed spermatogonia.

At 2 hours post radiation, considering all dosages used, the lowered incidence of frequency of the cells never fell below the normal minimum. The cells appear normal. There is some edema, which is consistent with the reports of many observers that a period of edema follows x-irradiation (1). The period from 1 day on shows a progressive decrease in frequency with

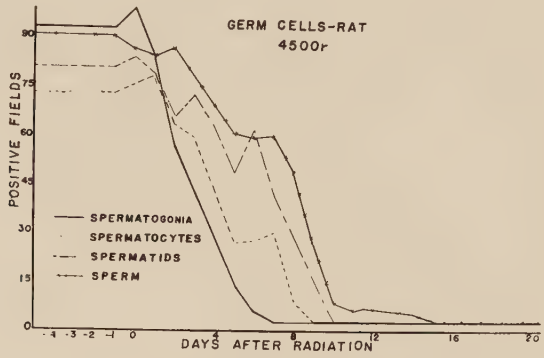


Fig. 8. Incidence of frequency of each type male germ cell at intervals following direct irradiation, 4 500 r.

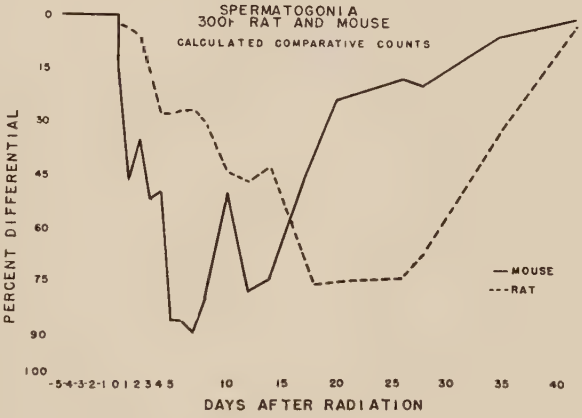


Fig. 9. Comparison of the same dose 300 r, direct irradiation, on incidence of frequency of spermatogonia at intervals post radiation. The normal incidence is taken as 0. Reduced incidence is then calculated in per cent.

increased dosage. The slope of the curve representing this decrease appears to be the same regardless of dose. For 138 r, the lowest incidence of frequency has been reached in about 6 days. With 2 000 r, the lowest incidence of occurrence of any given cell, as observed in 100-cross sections of tubules representative of both testes, may be designated by the term "low point." The average low point for 2 000 r is 1, the average for 138 r is 43. These figures can be expressed in per cent using 77 (the incidence of frequency for non-radiated animals) as the equivalent of 100 per cent. If we now include the data taken from the previously reported doses of 300 r, 1 440 r and 5 050 r, it can be seen that there is progressive decrease of tubules showing spermatogonia with increase of dose. This is expressed in per cent (Table II).

If all of the spermatogonia have not been destroyed there is, it may be assumed, a period of recovery; fig. 12 shows a tubule where spermatogonia

Table I

Per cent decrease in occurrence. Low Point Post Radiation.

	Spermatogonia	Spermatocytes	Spermatids	Sperm
138 r	45	61	34	0
300 r	86	84	85	93
1 440 r	97	97	98	97
2 000 r	99	99	99	99
5 050 r	100	100	100	100

Table II

Per cent of total recovery. 12 Weeks Post Radiation.

	Spermatogonia	Spermatocytes	Spermatids	Sperm
138 r	100	100	100	100
300 r	100	86	83	78
1 440 r	65	57	55	40
2 000 r	44	43	46	43
5 050 r	0	0	0	0

have persisted beyond the low point period apparently morphologically unchanged. In 28 days the animals treated with 138 r show complete recovery. The animals treated with 2 000 r show germ cells in only a few cross sections of tubules after 12 weeks. The tubules that do have germ cells, however, do not show any histological or cytological damage. This indicates that with increased dosage a greater volume of the testes has been made incapable of producing germ cells.

Figs. 6 and 7 show the effect on all four of the germinal elements after the doses of 138 r and 2 000 r. These demonstrate that the spermatogonia consistently are affected first, followed in time by the spermatocytes, spermatids and sperm. Apparently this succession of events is without regard to dose. Considering the curves for spermatogonia and spermatocytes, it will be noted that the slope of the curves and the low points are approximately the same. However, the spermatids do not reach as low a frequency of appearance. The frequency of appearance of sperm do not differentiate from the normal.

The higher doses, as shown by 2 000 r, emphasize some aspects of the biological effect that are not clearly apparent at 138 r. For example, the

frequency of appearance of each germ cell drops to a nearly equivalent low point; 18 to 20 days elapse between the time the spermatozoa reach this stage and the time the low point is indicated in the sperm. There is marked evidence of liquefaction necrosis post radiation, particularly after the higher doses, fig. 13.

In the study of the mouse, the addition of the two dose levels to results previously reported, completes a direct radiation series ranging from non-sterilizing doses which permit a complete recovery to these doses which cause sterility.

The results of direct x-irradiation on rat testicles are indicated in figs. 2 through 5 and fig. 8. The data presented for a comparison of rat and mouse are based on material subjected to the same experimental method. That is, the same rack and the same factors of radiation were used. Because there is a difference in size between the rat and mouse testis, the question might arise whether size was a factor to be considered in our counts. In our experience there is no marked variation in counts made from different areas of normal testes either in rat or mouse. The small testis of the mouse is at the maximum distance from the point of radiation. The proximal surface of the mouse testis is approximately 1.5 cm farther away from the target of the x-ray than the same locus for the rat testis. Also the size of the rat testis makes it necessary to consider the possibility of scatter radiation. In a preliminary screening test, the testes of two rats 7 days post radiation were counted. In this case the testes were identified as near areas (that half of the testes nearest the target) and deep areas (that half of the testes the greatest distance from the target). Counts indicated that there was no significant difference within the limits of biological variation. This suggests that the variation in counts noted at 300 r for mouse and rat were not associated with difference in size.

The rat control animals show a high incidence of frequency of appearance for each germ cell. Our quantitative method applied to rat testes at intervals post radiation gives the same general pattern of response as was found for mouse testis. At 16 days after the acute dose of 300 r only 24 of every 100 cross sections will reveal spermatogonia. This is the low point for frequency of appearance. At later intervals the frequency rapidly approaches normal and is entirely normal by 42 days. The studies on spermatogonia shows there is marked biological variation from animal to animal.

As one can see from the graphs figs. 2 through 5 and fig. 8 each one of the germ cells goes through a period post radiation of decreasing frequency, a low point and a period of recovery, but at progressively later periods from spermatogonia to sperm respectively. Counts made on animals later than 42

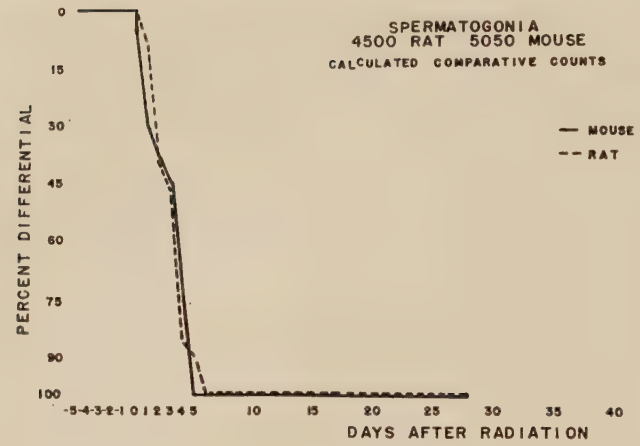


Fig. 10. Comparison of 5 050 r, mouse, irradiation on incidence of frequency of spermatogonia and 4 500 r, rat. The method of calculation is the same as for fig. 9.

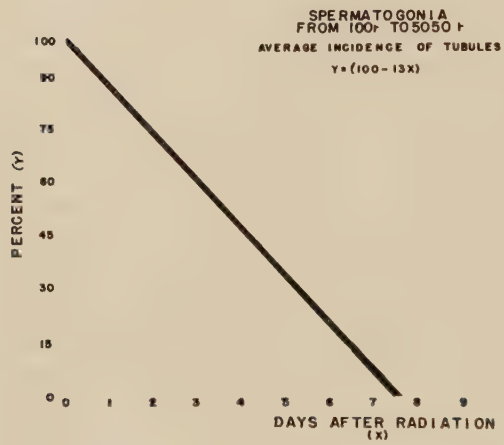


Fig. 11. Incidence of frequency of spermatogonia in cross sections in per cent (Y) plotted against days after radiation (X).

days post radiation show a complete return to normal frequency of all germ cells indicating that no permanent damage has been done to the testes by an acute dose of 300 r.

An examination of fig. 5 for sperm would suggest that it was very similar to normal. Actually, however, it is assumed that it does have the same sequence of events as described above. The counts do not indicate that the sperm at 40 days, the low point, have decreased much. The low point for sperm, however, is 40 days while that for spermatogonia was 16 days.Thus there is an interval of 24 days when a cycle of production of germ cells including sperm has been going on. This allows a sufficient interval for some

new sperm to have been formed thus accounting for the near normalcy of frequency of sperm.

The graph—fig. 8—for 4 500 r show that sterilization has been produced. All the germ cells, whether resting or in mitosis, have disappeared as a result of the radiation. The time of disappearance for each type germ cell is progressively later from spermatogonia to sperm, but all germ cells disappear in 6 to 10 days.

DISCUSSION

The submitted quantitative data based on studies of the effect of direct x-irradiation on mammalian testicles reveals two major features that are common to both rats and mice and presumably would be true of humans, namely that there are factors which are 1) regardless of dose and 2) dependent on dose.

We can use our data to estimate dosage effect at a given interval post-radiation. After 24-hours, there is a decrease in the incidence of frequency for each germ cell. This was calculated for spermatogonia by plotting the per cent of incidence of frequency of spermatogonia in cross sections against days after radiation. This is a straight line function when drawn on cross section paper (fig. 11).

In the nonsterilizing doses it has been postulated that there is a residuum of germ cells not permanently affected by the radiation (5) and that in time these cells will differentiate into sperm. This theory gains some support from our data. For example, at 138 r, the sperm do not show a significantly decreased incidence. There is a slight decrease which is suggestive of a biologic effect, but the replacement by less differentiated germ cells presumably has kept the incidence nearly normal, thus masking the maximum biologic effect.

At the higher dose, 2 000 r, replacement has not taken place and therefore the slope of the curve representing biologic effect appears the same for the sperm as for the spermatogonia. When we consider that regardless of dose each type germ cell reacts at the same rate but at later intervals, the question of relative sensitivity is raised. The question needs further study.

This mouse series reveals certain factors dependent on dose: a) It has been necessary to give more than 2 000 r, direct x-irradiation to cause sterility. This exceeds the over-all lethal dose. b) It is indicated that for each dose there is a specific reduction in the incidence of frequency. In the small dose, 138 r, it is not clear by our method whether the decrease in incidence means an inhibition of mitotic activity and the loss of cells in mitoses as suggested

by Eschenbrenner and Miller (3). In the larger dose, 2000 r, the incidence has been reduced 99 per cent for each germ type. This suggests a general loss of all phases of division in the spermatogonia and spermatocytes and the stages of differentiation from spermatids to sperm. Preliminary observations made on irradiated tissue show definite liquefaction necrosis. It is assumed that this represents general destruction for those cells in this area. Coalescence of cells, in this case spermatids, into multinuclear units in various stages of liquefaction necrosis, can be seen, fig. 13. Gardner-Boddaert (6) gives a similar description of general destruction of cells in testis following subcutaneous injections of the synthetic estrogen tri-p-anisyl chloroethylene. c) There is reduction in total recovery with increase in dose. Total recovery is measured by the number of cross sections of tubules showing germ cells of each 100 observed per testis. For example, after 12 weeks post radiation 2 000 r, only 44 cross sections of 100 will show spermatogonia. This can be expressed in per cent recovery, Table II.

The decrease of frequency of occurrence of any germ cell from the normal can be expressed in per cent, Table I. This has been calculated from data taken at that time interval when the frequency was at minimal.

The effect of direct x-irradiation on the testes of mice and rats gives similar results which are more apparent at the higher dose range, fig. 10.

The relative differences between the rat and the mouse based on our method of determining the frequency of occurrence of germ cells in tubules have been plotted, fig. 9.

In order to make the curves comparable, both the mouse and the rat data for incidence of the frequency of spermatogonia were plotted as per cent on the abscissa taking zero as the normal frequency. At 300 r, it is observed that the slopes of the curves for mice and rat are not the same, recovery for mice starts at an earlier interval but by 42 days both have returned to normal. Fig. 10 is a similar comparative count plotting per cent decrease against intervals after radiation for spermatogonia. The two curves are almost identical.

The effect of radiated testis on endocrine function has not been fully established as has been discussed by Liebow, Warren and Decoursey (8). Also, the matter of possible induced radio-resistance of the germ cells needs investigation.

SUMMARY

Quantitative data are presented to show biological effects on rat and mouse testicles after direct x-irradiation. The method of quantitative random

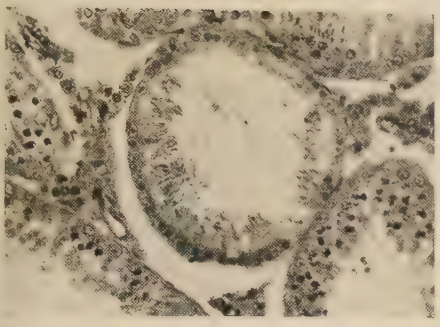


Fig. 12.

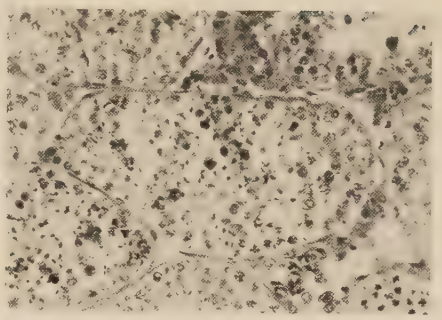


Fig. 13.

Fig. 12. Cross section of tubule mouse testis, 21 days post radiation, 300 r. Peripheral spermatogonia are present. (× 500.)

Fig. 13. Cross section of tubule, mouse testis, as it appears 3 days post radiation, 2 000 r, showing liquefaction necrosis. (× 500.)

sampling indicates a predictable and reproducible series of events some of which are regardless of dosage and others that are dependent on dose.

Regardless of dose: (1) There is no lowered incidence of frequency of any germ cell in the first 24 hours. (2) After 24 hours there is a calculable rate of decrease of incidence. This decrease is first noticed in spermatogonia followed progressively at later intervals by spermatocytes, spermatids, and sperm. (3) There is a period post radiation at low doses when there is marked biological variation.

Dependent on dose: (1) 4 500 r is a sterilizing dose in rats. This corresponds to 5 050 r for similar effect on mice. (2) All doses show a similar pattern of response. For each kind of germ cell, at whatever dose, there is indicated at intervals postradiation where the incidence of frequency of appearance is minimal. (3) There is a dosage level beyond which there is not total recovery.

The biologic effect as noted by direct x-irradiation for rat and mouse was similar.

A quantitative method is utilized which reveals a predictable biologic response and thus has the attribute of permitting prognosis of recovery.

This work was supported by Grant C-1047 from the United States Public Health Service and by the Department of Public Health, Pondville Hospital.

A preliminary report of this paper was presented on December 30, 1950 at the American Association for the Advancement of Science, Medical Section, Cleveland, Ohio.

REFERENCES

1. Bloom, W., Histopathology of Irradiation from External and Internal Sources, McGraw-Hill Book Co, Inc., New York, Toronto, London, pp. 40, 55, 568, 1948.
2. Eschenbrenner, A., and Miller, E., *Arch Path.*, **6**, 736 (1950).
3. Eschenbrenner, A., Miller, E., and Lorenz, E., *J. Natl. Cancer Inst.*, **6**, 343 (1946).
4. Fogg, L., and Cowing, R., *Cancer Research*, **1**, 23 (1951).
5. —— *ibid.*, **2**, 81 (1951).
6. Gardner, W. U., and Boddaert, J., *Arch. Path.*, **50**, 750 (1950).
7. Lea, E., Actions of Radiation on Living Cells, Cambridge University Press, London, p. 364, 1947.
8. Liebow, A., Warren, S., and Decoursey, E., *Am. J. Path.*, **25**, 919, 924, 928 (1949).
9. Warren, S., *Arch. Path.* **35**, 121 (1943).

ON THE COMPONENTS AND STRUCTURE OF THE HUMAN RED CELL MEMBRANE[1]

M. MOSKOWITZ[1] and M. CALVIN

Department of Chemistry, University of California, Berkeley, Calif., Department of Microbiology, Yale University, New Haven, Conn., and Blood Grouping Laboratory, Boston, Mass.

Received April 10, 1951

In the course of a study on the concentration of the Rh factor from human red cells (18), observations were made on the various fractions separated from red cells which provide information of value in elucidating some aspects of the structure of the red cell membrane. The Rh factor was found to be extremely labile and the gentle fractionation procedures employed permitted the preparation of red cell fractions which can be considered to be undenatured, at least insofar as their solubility characteristics are concerned. Also, as the Rh activity of the different fractions were assayed, an indication of the extent to which the different components were separated was available, for the Rh factor was found to be associated with a single fraction. The A and B blood group factors were also found to be associated with this fraction when red cells of the respective type were fractionated.

The picture of the red cell membrane that is suggested by this work is as follows: Long, fibrillar material, called elinin, is arranged in a manner such that the fibrils lie side-by-side and parallel to the surface of the cell. These fibrils are joined together by the ether-extractable lipids to form the framework of the membrane, which is referred to as stromin.[2] Hemoglobin, a fraction called S protein, and possibly other components, are associated with stromin to form the red cell membrane.

The preparation of elinin and S protein (19), and some physical studies on elinin (6) have been reported. Water solutions of elinin are perfectly clear with transmitted light but show marked Tyndall scattering. Elinin has a relatively high particle weight, about forty million, and the particles are asymmetric, undoubtedly being rod shaped. Streaming birefringence is easily demonstrated by squirting a water solution with a capillary pipette between crossed polaroid filters. It is a lipid-carbohydrate-protein complex containing about eight per cent nitrogen and 1.7 per cent phosphorus; about 25 per cent of elinin is extractable with alcohol-ether. The Rh, A and B blood

[1] Present address: Purdue University, Lafayette, Indiana, U.S.A.

group factors reside exclusively in the elinin fraction. The S protein fraction is soluble in water above pH 7, and precipitates at pH 6.4. It is much lighter in weight than elinin, contains about 15 per cent nitrogen and is devoid of the Rh, A or B factors.

The experiments which led to the separation of elinin, and the preparation of stromin, will be described here, and the evidence that suggests the above picture of the red cell membrane will be discussed. The experimental work is presented essentially in the sequence in which it was carried out, inasmuch as this permits a description of the properties of the fractions in the various stages of separation.

What is often regarded as the red cell membrane has been previously referred to as ghosts, stroma, stromata, post-hemolytic residue, etc. (24). In the description of the experiments the residue remaining after the red cells are hemolysed and after it has been subjected to various treatments will be referred to as stroma whether or not the material comprising the residue is present in the form of the intact 'ghosts.' The term ghost will be used only when it is desired to emphasize that the hemolytic residue under consideration is composed of intact ghosts. There is some confusion in the literature regarding the terminology used to describe red cell components, especially in regard to the usage of the term stromatin. As the results of this work permit a better understanding of the nature of some of the red cell components, a more exact definition of the various terms will be given in the discussion.

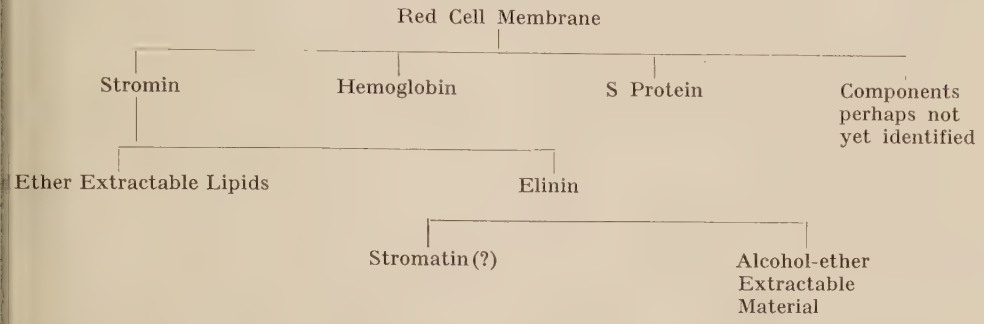
EXPERIMENTAL

1. *The Effect of Organic Solvents on Stroma:* Initially an unsuccessful attempt was made (18) to extract the Rh factor with organic solvents. The extractions were performed on stroma prepared according to the method of Calvin *et al.* (4). The final precipitate was dried from the frozen state in a lyophilization apparatus. After a sample of stroma was extracted, the solvent remaining on the extracted stroma was removed by means of reduced pressure. The extracted stroma was suspended in a phosphate buffer ($\mu = 0.15$; pH 7.7) to test for its Rh activity.

It was found that when dry stroma was extracted with 95 per cent ethyl alcohol at 1° C, the Rh activity was destroyed. After removing the alcohol, the stroma had the consistency of hard rubber and was completely refractory to suspension in the buffer solution. When a water suspension of stroma at pH 7.7 was extracted with ether or acetone at 1° C or by bringing the temperature of the mixture to below minus 30° C, the stroma appeared to be aggregated and the Rh activity was destroyed. On suspending these preparations in buffer it was observed that the stroma settled out of suspension on standing. When unextracted stroma was suspended in buffer, it formed a fine suspension which appeared homogeneous; that is, no particulate matter settled out on standing. Thus it appeared that the above treatments had

TABLE I

Outline of the Components of the Human Red Cell Membrane.



altered the stroma in some manner which caused it to aggregate. If dry stroma was extracted with dry ether in a soxhlet apparatus, the Rh activity was not destroyed and the extracted stroma did not settle out of suspension on standing in buffer solution. In fact, these suspensions of extracted stroma appeared much clearer than those prepared from unextracted stroma, and most of the extracted stroma appeared to be in solution. When the manner of preparing the stroma was changed, as will be described, extracted stroma preparations were completely soluble in water. These observations indicate that the usual procedures used in extracting stroma preparations, such as alcohol-ether extractions of wet preparations, have a denaturing action on the stroma which alters its properties.

2. *The Quantitative Removal of Stroma by Centrifugation of the Hemolysate:* Packed red cells, prepared as described previously (19), were hemolysed with 10 volumes of water (pH 7.3) and centrifuged at 20 000 G in an angle rotor for 2.5 hours. The sediment contained all the Rh activity. The supernatant fluid was water-clear[3], and further centrifugation of the supernatant fluid at 20 000 G for 3 hours yielded no trace of sediment. The sediment was suspended in water to the original volume and centrifuged at 20 000 G for 1 hour. This was repeated once again with the resulting supernatant fluid being practically colorless. The sediment was again suspended in water to the original volume, the pH adjusted to 9, and the suspension centrifuged at 20 000 G for 1 hour. The supernatant fluid obtained was as colored as that obtained with the first washing at pH 7.3, and two more washings were necessary to achieve a colorless supernatant fluid. The supernatant fluids were water clear and the final sediment, which was still quite reddish, contained all the Rh activity. These results showed that the hemolysate could be divided into 2 fractions: a heavy one which contained the Rh factor (and the A and B factors as was subsequently demonstrated) and a light one which was devoid of the Rh factor (and the A and B factors). They also showed that more hemoglobin was released as the pH was increased.

3. *The Preparation of Solutions of Extracted Stroma:* In order to obtain large amounts of stroma to fractionate, the hemolysate was centrifuged on a 'supercentrifuge' as previously described (19). The ghosts were washed twice with water (pH 7.3)

by centrifugation in an angle rotor at 100 000 G for one hour, and then washed with water at pH 9 until a colorless supernatant fluid was achieved. The supernatant fluids were water-clear and contained no Rh activity. The final sediment was dried from the frozen state and extracted with ether in a soxhlet apparatus. The extracted stroma was suspended in water at pH 9, and in a few hours most of the extracted stroma was in solution.

The results obtained with this procedure, insofar as the physical characteristics of the material were concerned, were not constant. The following obvious variations were noted: 1) The color of the sediment obtained after the final washing at pH 9 varied from a light pink to a definite red. 2) The color of the supernatant fluid obtained on successive washings varied. The washings of the sediment of the 'supercentrifuge' were carried out over a period of two or three days. When two washings were made at pH 9 on a single day, the supernatant fluid obtained from the second washing was invariably much lighter than that obtained from the first washing. However, if a third washing was made at pH 9 the next morning, the supernatant fluid was often as colored, if not more colored, than the supernatant fluid obtained from the first washing on the previous day. 3) The extracted stroma did not always go into solution in water, and none of the preparations were completely soluble in water. Of those preparations that did go into solution, from ten per cent to fifty per cent of the Rh activity could be removed by centrifugation at 20 000 G for 15 minutes.

These variations were found to be interrelated. It was observed that the solubility of the extracted stroma was correlated with the final color of the washed stroma, which in turn was related to the increased color of the supernatant fluids as described above. The lightest preparations were the least soluble; the darkest preparations were the most soluble. The colored material[4] which was removed during the washings apparently protected the stroma in some manner, and it was therefore able to go into solution after extraction. As a result of these observations, the sediment obtained from the 'supercentrifuge' was dried directly with no washing, and it invariably went completely into solution after extraction.

When 0.5 per cent water solutions of extracted stroma at pH 7.5 to 9 were centrifuged at 100 000 G for one hour in an angle rotor, the sediment contained all the Rh activity and the colored supernatant fluid was waterclear. On suspending the sediment in water, a brownish solution was formed manifesting the same physical properties as elinin. The further development of this method of separating the fraction that contained the Rh factor resulted in the present procedure for preparing elinin (19).

4. *Preparation of Stromin:* A 0.5 per cent suspension of stroma (prepared by drying the 'supercentrifuge' sediment with no washing) was made in water. After standing for one hour (pH 7.3), it was centrifuged at 20 000 G in an angle rotor for one hour. The sediment was washed once with no pH adjustment, and then twice at pH 9 by centrifugation at 20 000 G for one hour. The washed sediment, called stromin, appeared creamy white. The supernatant fluids were all water-clear, and the supernatant fluid obtained after adjusting the suspension to pH 9 was darker than that obtained at pH 7.3. S protein was precipitated from the supernatant fluids at pH 6.4. A one per cent suspension of stromin appears opalescent and manifests 'streaking'[5] and streaming birefringence. When stromin was dried from the frozen

state, it appeared white; after the dried material was extracted with ether—in the same manner stroma is extracted in the preparation of elinin (19)—it did not go into solution in water at pH 9.

Microscopic examination of stromin preparations showed that they consisted almost exclusively of rod shaped particles. In one dried preparation they ranged in length from five to eleven microns and were about 0.3 to 1.3 microns wide. Dark field examination of the suspensions shows rod shaped particles of varying length and width and, as they moved about in the fluid, the wider ones were observed to be ribbon shaped or platelets.

DISCUSSION

The Structure of the Red Cell Membrane

In developing the picture of the red cell membrane described in the introduction, the membrane will be considered as the outer portion of the red cell which plays a role in determining the passage of substances into and out of the cell. It may appear, in view of current concepts of the nature of the membrane, that the stromin fraction constituted the membrane. The evidence suggests, however, that some hemoglobin and S protein are so closely associated with stromin that they may be responsible for some of the permeability properties of the red cell, and they therefore may be part of the membrane. And, of course, it is possible that there are other components in the membrane that have not yet been recognized.

The framework to be described here corresponds essentially to the material which is defined in Webster's dictionary (29) as stroma, *e.g.*, "the colorless framework of the red blood corpuscle." However, as the term stroma has come to be applied to red cell fractions prepared in different ways, such as has been done here to facilitate the presentation of the experiments, in order to define the fraction under discussion the material composing the framework is referred to as stromin. Another reason for differentiating stromin from stroma is because stroma is often conceived of as "that part of the red blood corpuscle which remains after the hemoglobin has been removed" (7), and, as there are probably other materials in stroma besides stromin — such as S protein— stromin would then be but one of the components making up stroma.[6]

The framework of the membrane is conceived as surrounding the cell somewhat like the skin of a balloon. Electron micrographs of hemolysed red cells (30) demonstrate the presence of such a structure. With the method described for preparing[7] stromin, the framework is broken up into fragments of the size and shape noted. When stromin is extracted with ether (in the presence of the 'colored material'), the smaller rods of elinin are set free. It appears that the rods of elinin are held together by the ether-extractable lipids. Electron micrographs of elinin preparations (6) showed the presence of rods ranging in length from about 2 500 to 10 000 Å, with the narrowest rods being about 125 Å in width. The fact that rods of this size could only lie on the long axis of the stromin particles suggests that they are arranged parallel to the surface of the cell. Although it does not show up in the reproductions, some of the rods which are wider than 125 Å are observed to be made up of the narrower rods lying side-by-side. The presence of these groups of rods may be explained on

the basis of incomplete extraction of those particular complexes. Also, a close examination of the original prints shows that some of the rods manifest a periodic increase in optical density at distances of about 125 Å, appearing as if the rods were polymers of these smaller units. Thus the separation of elinin in the form of rods is probably a consequence of the method used in separating them; the basic structural component of the membrane may be the 125 Å units (*vide infra*). The rods of elinin, in turn, are probably portions of long fibrils within the membrane. Schmitt *et al.* (26) studied red cell membrane preparations with a polarizing microscope, and they appeared to be made up of protein particles with their long axis parallel to the surface and interspersed lipid micelles oriented radially. Wolpers (30) examined hemolysed red cells in the electron microscope after they were extracted with an organic solvent, and observed that the membrane contained fibrillar material, appearing like long strings lying side by side on the surface of the cell. Elinin apparently corresponds to what these workers observed as the fibrillar portion of the membrane. The width of the individual rods of elinin are about the same as the fibrils observed by Wolpers; their spatial arrangement within the membrane corresponds to the arrangement observed by Schmitt.

The fibrillar component of the red cell membrane has been heretofore presumed to be of protein nature. However, if the fibrils are made up of elinin, they are then not exclusively protein in nature. Approximately fifty per cent of the lipids of the membrane[8] can be extracted with ether, extraction with alcohol-ether being necessary to remove the remaining lipids. This was observed in extraction experiments (18) and by Parpart and Dziemian (21). The lipid which remains after ether extraction is thought of as existing in the form of a lipo-protein complex (5), with a protein component of the membrane. As elinin is obtained after ether extraction, it contains the alcohol-ether extractable lipids. Twenty-five per cent of elinin is extractable with alcohol-ether. Elinin also contains carbohydrate and therefore should be regarded as a lipid-carbohydrate-protein complex. Thus the 'protein' component observed by Schmitt *et al.* (26) also contained lipid and carbohydrate, and the lipid which he observed probably comprised roughly fifty per cent of the lipids of the membrane. The possibility exists that elinin consists of a protein core surrounded by lipid, but attempts to remove the lipids present in elinin invariably resulted in the loss of streaming birefringence and the denaturation of elinin.

In the course of preparing what are considered membrane preparations, most workers seek to obtain a product as free from hemoglobin as possible because hemoglobin is assumed to be a contaminent, but to the author's knowledge there is no evidence which shows that hemoglobin is not an integral part of the membrane. The experiments cited here do not demonstrate the presence or absence of hemoglobin in the membrane, but they do suggest that it may be part of the membrane. The concept of hemoglobin constituting part of the red cell membrane is not new. Adams (1) postulated the existence of a 'stromatin-hemoglobin complex' in the intact red cell on the basis of the lack of absorption in the 4 100 Å region with red cells and mixture of 'stromatin' and hemoglobin. (This has been disputed: 16, 25.) In stating that hemoglobin constitutes part of the membrane, the implication that all the hemoglobin of the red cell is contained in the membrane — a view set forth by Bürker (3) — is not intended. What is being suggested is that the hemoglobin which is associated with the stromin in the ghost after the red cells are hemolysed and washed may be

an integral part of the membrane. This hemoglobin perhaps corresponds to the 'surplus' hemoglobin found by Ponder (22) in the red cell ghost.

The reason for stating that hemoglobin may form part of the membrane is simply because hemoglobin is found to be so intimately associated with the stromin. When red cells are hemolysed in water, one of the gentlest methods of removing the ghosts is by centrifugation. After the ghosts are washed in water, with no pH adjustment in the centrifuge, until a colorless supernatant is achieved, they still contain hemoglobin.[9] (This is also observed when the hemolysate is made up to a concentration of 0.15 molar with sodium chloride and centrifuged in the same manner.) The hemoglobin which remains cannot be considered as being contained within the ghost, for the phenomenon is observed during the preparation of stromin when only fragments of the ghost are present. If the washed ghosts are suspended in water and the pH increased to 9, a dark red supernatant fluid is obtained after centrifugation. When these ghosts are resuspended in this colored supernatant fluid, and the pH adjusted to 7.3, the supernatant fluid obtained on centrifuging this mixture is as colored as it was at pH 9. This indicates that hemoglobin is closely bound to the stromin, and alkali breaks up this association. Whether or not such an association actually exists in the intact red cell is not decided by the experiment cited, as it could have occurred after hemolysis. But the fact that the hemoglobin did not reunite with the stromin when the pH was lowered to 7.3, as described above, suggests that the association is not an artefact of the preparative technique.

Drabkin (8) has estimated that if hemoglobin were a constituent of the red cell membrane, there would not be room for more than two per cent of the hemoglobin of the red cell. The yields of ghosts obtained in the 'supercentrifuge,' and at 20 000 G in the angle rotor, cannot be considered quantitative, due to the incomplete removal of ghosts in the former and the loosely packed sediment, with the concomitant occlusion of hemoglobin, in the latter. In an experiment to determine the quantitative yield of ghosts, the hemolysate was centrifuged at 100 000 G for two hours. The sediment was tightly packed and the water-clear supernatant fluid could be removed without disturbing the sediment. The sediment was dried and the yield of ghosts under these conditions was 1.65 g per 100 ml packed red cells. About 0.55 g of the sediment could be accounted for on the basis of the ether extractable lipids and elinin. The remainder consisted of hemoglobin, S-protein, and perhaps other constituents of the membrane. Even if all the remainder was hemoglobin, this amount of hemoglobin would constitute just about three per cent of the hemoglobin of the red cell. However, some of the hemoglobin which was found in the sediment would be removed on further washing of the ghosts, and therefore the amount of hemoglobin which is associated with the membrane is less than three per cent of the hemoglobin of the red cell. Also, it is not necessary to have all the hemoglobin in the membrance, as was figured in Drabkin's calculation, as a surface layer; it may be in the interstices and on the inside surface of the membrane.

The reasons for regarding S protein as part of the membrane are the same as those just given for hemoglobin, inasmuch as S protein was present along with the hemoglobin in the colored supernatant fluids. The distribution of S protein in the red cell may not be confined solely to the membrane; perhaps some of it is located in the interior of the cell along with the hemoglobin. There is no clue to the manner in which hemoglobin and S protein are associated with the stromin in the membrane.

However, there are indications that some hemoglobin and S protein are associated with elinin. This is brought out by the fact that when solutions of extracted stroma, prepared as described in the preparation of elinin (19), are centrifuged at pH 7.3 until a colorless supernatant fluid is achieved, some 'colored material' remains with the sediment (elinin), and this is removed, as with unextracted stroma, at pH nine.

The question as to whether or not there is an interior structure in the red cell has not been discussed, except to mention that S protein may be present in the interior, because our findings provide little information on the subject. It is felt that a relatively thin membrane surrounding the red cell need not be incompatible with the presence of an interior structural organization, and if an interior structure is present it need not necessarily detract from the hypotheses presented here. We have not demonstrated that all the elinin obtained from red cells is present in the surface membrane, but from the work which has been discussed it would appear that such is the case.

This discussion of the structure of the red cell membrane has of necessity been speculative. There are certain unanswered questions, such as an analysis of the different lipid fractions, which should not be difficult to answer, and it is hoped that such studies will eventually be carried out. The prime purpose of the work, however, was to produce a concentrated Rh fraction to be tested clinically (10), and our efforts were directed toward this end; the observations relating to the structure of the membrane were incidental to the main work. Nevertheless, it is felt that these observations in their present state of development should be of value to workers interested in the nature of the red cell membrane.

The properties of the red cell membrane components under various conditions

In this section of the discussion the observations on the size and stability of the membrane components will be briefly described, and their relation to the fractions obtained by other workers will be discussed.

The Breakdown of the Red Cell Ghost and Its Components: In the experimental part a method is described for breaking the red cell ghost into fragments the size and shape of stromin. Why does not the ghost always break down into such particles? There are some indications to the answer of this question, although much remains obscure.

Working with samples of cells from different individuals, differences have been observed in their behavior after hemolysis. Occasionally when cells of one person were hemolysed and centrifuged in an angle rotor, without pH adjustment (pH 7.3), as described in Part 2 of the experimental work, a water-clear supernatant fluid was not achieved after 2.5 hours. Further centrifugation for three hours did not clear up the supernatant fluid. When the cloudy supernatant fluid was removed and centrifuged for three hours as above, it remained cloudy and no trace of sediment was observed. These supernatant fluids, in contrast to those which were water-clear, contained some Rh factor. In these instances some of the ghosts broke down into extremely small particles. It would appear that the ghosts obtained from the red cells of different individuals vary in their stability, and if observations on the break-

down of ghosts are made on but one or two samples of red cells, the results may not be representative.

Stroma prepared at an acid pH, dried, extracted with ether, and put in solution at pH 9, is also difficult to remove by centrifugation. In a series of experiments the major portion of the dissolved stroma was not removed after centrifugation at 100 000 G for hours. The supernatant fluid was not water clear and contained most of the Rh factor. In the discussion on the structure of the membrane it was suggested that elinin may be composed of 125 Å units. The relatively small size of the particles remaining in the supernatant fluid, and the lack of streaming birefringence in such solutions, indicates that the particles may consist of these 125 Å units or smaller multiples of them than are present in the elinin preparations. The breakdown of ghost probably depends on such factors as ratio of water to ghosts, pH, temperature, etc.

Ponder (24) has recently reported on the breakdown of ghosts as a function of the time they are hemolysed and of the temperature, and described how they start changing within 20 minutes after being hemolysed at room temperature. The changes he describes may be responsible for the apparent differences in stroma preparations prepared from cells that were hemolysed for a period of hours (*vide infra*); they also explain, as he discusses, why it is not possible to obtain clear supernatant fluid when hemolysed red cells are centrifuged under certain conditions. We mentioned in the experimental portion that we centrifuged ghosts over a period of days and still obtained water clear supernatant fluids. If the breakdown of the ghosts progressed, as described by Ponder, this would not be possible. Perhaps there is a substance in the hemolysate which incites the breakdown of the ghosts and once the ghosts are removed from the hemolysate this breakdown does not occur.

The Component that Stabilizes Elinin: In Part 3 of the experimental section it was mentioned that the 'colored material' prevented the denaturation of elinin during the extraction process. The absence of this 'colored material' from stromin preparations is the reason they do not go into solution after being extracted with ether. The presence of the 'colored material' also affects the properties of elinin. When a water solution of extracted stroma, prepared in the course of separating elinin (19) is adjusted to pH 5.5, a precipitate forms. This precipitate contains the elinin. If the pH is again raised above neutrality, or lowered below 5, the precipitate completely redissolves. If the solution is frozen and thawed, no precipitate forms, and when this solution is dried from the frozen state, it readily dissolves in water. This behavior is in contrast to that of the elinin which is separated from such a stroma solution. Solutions of elinin are irreversibly precipitated on bringing the pH to 5.5 or on freezing and thawing the solution and, after being dried from the frozen state, they cannot be dissolved in water. The identity of the stabilizing component is not known.

The Relationship of the Components to Those Described by Other Workers: Red cell stroma is usually prepared by precipitating it from the hemolysate at an acid pH. If the stroma is precipitated and washed without the addition of salt, it contains a relatively large amount of hemoglobin; if salt is added during this procedure, more hemoglobin is dissociated, and the stroma contains proportionately less of it. The stroma prepared without the addition of salt undoubtedly contains S protein and, as S protein is soluble in salt solutions at the pH most often used in preparing stroma (about 5.5), stroma prepared in the presence of salt may or may

not contain S protein. Assuming that stroma prepared at an acid pH in the presence of salt contains little, if any, hemoglobin or S protein, it would then be composed mainly of stromin.

Jorpes (15) prepared stroma by precipitating and washing it with a phosphate buffer at pH 5.5, and extracted it with alcohol-ether. The insoluble residue which was obtained after extraction he called stromatin, and it is usually regarded as the protein component of the red cell membrane. As stromin is composed of elinin plus the ether extractable lipids, and as alcohol-ether extraction of stromin would remove both the ether extractable lipids of stromin plus the lipids of elinin, stromatin therefore corresponds to the protein component of elinin (Table I). If the stroma prepared according to the method of Jorpes contained S protein, or some protein fraction as yet unidentified, it would of course be present in stromatin in addition to the protein component of elinin.

On the basis of its solubility characteristics, stromatin has been likened to the scleroproteins. The solubility characteristics of stromatin were apparently brought about as a result of the method used in preparing it and are not representative of the physical state of stromatin in the intact cell. The relationship of stromatin to elinin and stromin has already been discussed. Elinin preparations are completely soluble in water, but when they are exposed to alcohol-ether they assume the solubility characteristics of stromatin. Stroma prepared according to the method of Jorpes forms a fine suspension at an alkaline pH and much of it goes into solution; after drying the precipitated stroma from the frozen state and extracting it with ether, it goes into solution in water at an alkaline pH. Inasmuch as stroma is soluble after ether extraction, and in view of the known denaturing effects of alcohol, alcohol-ether mixtures probably denature the protein and give rise to the solubility characteristics of stromatin.

If stroma is prepared by centrifugation of the hemolysate without adjusting the pH (pH is 7.3–7.35), it contains hemoglobin, S protein and stromin. Less hemoglobin and S protein are associated with stromin in this case than when stroma is prepared by acid precipitation. This is brought out when stroma prepared by acid precipitation is adjusted to a pH above seven and centrifuged until a relatively clear supernatant fluid is obtained. The supernatant fluid is red, due to the hemoglobin that dissociated, and when it is brought to a pH below 6.5 the S protein it contains precipitates. Thus the hemoglobin and S protein are dissociated from the stromin to a greater extent at the alkaline than at the acid pH. Calvin *et al.* (4) fractionated hemolysates in the manner described above and separated two fractions which they called 'elinin' and 'stromatin.' The sediment obtained when the acid-precipitated stroma was brought to pH 7.7 and centrifuged was 'elinin' and the precipitate which formed when the supernatant fluid was acidified was called 'stromatin.' Their 'elinin' fraction corresponds to stromin plus some hemoglobin and S protein, and their 'stromatin' fraction, which is clearly not comparable to the stromatin fraction of Jorpes (15), corresponds to S protein.

Stern *et al.* (27) analyzed red cell hemolysates in the electrophoresis apparatus and observed three components, one of which was hemoglobin. The component called the *a* component, fits the description of stromin; whether or not their *b* component corresponds to S protein remains to be seen.

Boehm (2) prepared stroma, which he referred to as the stroma protein, in a manner

similar to that of Jorpes, and suspended it in lyotropic solvents. The resulting suspension was stringy, viscous, and manifested streaming birefringence. In the course of developing the methods of producing elinin and stromin, material similar to that described by Boehm, *e.g.*, of a stringy nature, was occasionally observed. But this phenomenon occurred only when stroma was handled without observing the precautions noted (19) or after exposure to organic solvents which had a denaturing effect on the stroma. The physical properties of the material prepared by Boehm probably were a result of the treatment to which it was subjected. Furchgott (12) attempted to repeat his work without success. He did observe, however, that under certain conditions the entire ghost would extend in the form of a long, threadlike particle which eventually broke up. If the break-up of the threadlike particle did not occur, and if a preparation consisted mainly of threadlike particles, such a preparation could manifest the properties of Boehm's preparations. Perhaps Boehm's technique provided conditions which prevented the break-up of these threadlike particles. If this is so, then he was dealing with what can be considered deformed ghosts.

Howe (13) describes the separation of 'lipid rich' (LR), 'lipid poor' (LP) and 'lipid free' (LF) fractions from human red cells. His LR fraction is apparently the same as stromin and the LF fraction the same as S protein. What he describes as the LP fraction is most probably a mixture of his LR and LF fractions and he suggests that this may be so. His work has added the valuable information that the influenza virus receptor is contained in the stromin component. He also describes a viscous, fibrous fraction which he obtained after dry ether extraction of a stroma preparation. In discussing the work of Boehm we mentioned that such fractions were often obtained in the course of our work, but were not obtained after the present method (19) for preparing elinin was adopted. A possible explanation for the presence of the fibrous material obtained by Howe is as follows: His fibrous fraction contained 8–9 per cent nitrogen which is the same as that contained in the elinin fraction (19); however, the fraction which he obtained by centrifugation of the extracted stroma preparation contained 12–13 per cent nitrogen. It would appear, on the basis of the nitrogen content of his fibrous fraction, that a small portion of his stroma preparation lost most of its 'colored material' and, as has been discussed, when 'stroma' contains little or no 'colored material' it is denatured by dry ether extraction. Perhaps in the course of preparing his original stroma preparation the hemolysate was handled in a manner that brought about the release of the 'colored material' from a portion of his preparation. In describing the preparation of elinin (19) it was emphasized that we hemolysed the red cells in 50 ml batches at 1° C and, although it was stated that the hemolysate was centrifuged within an hour after being made up, the average time the cells were hemolysed before being centrifuged was 30 minutes. This procedure was adopted because previously, when we had made up the hemolysate in a 6 liter batch and centrifuged it during the course of about 8 hours, the stroma did not go completely into solution after extraction. Thus the length of time his red cells were hemolysed before centrifugation, which was not mentioned by Howe, may be a factor in the production of the fibrous material he eventually obtained. This is but one of many possible reasons for the production of this fibrous material and it is mentioned in order to point out how seemingly insignificant variations in the preparation of stroma may affect the physical nature of the fractions obtained from it.

Mitchison (17) measured the width of red cell ghosts hemolysed in glycerol and suggests that width of the membrane is about 0.5 μ thick. This finding is somewhat incompatible with the concepts presented here and the hypothetical structure of the membrane he presents is at variance with our ideas as to its structure. He suggests that the membrane is composed of "long stromatin molecules arranged in looped bundles, like squibs or Chinese crackers." This picture of the membrane is based on the assumption that the material contained in the 0.5 μ thickness constitutes the membrane. However, what he may be measuring is the membrane plus some sort of interior structure.

The amount of "post hemolytic residue" one obtains depends on the way it is prepared. Thus, if the hemolysate in water is precipitated at pH 5.5, a much greater amount of material is obtained than if it is centrifuged without adjusting the pH. Further washing in both instances removes a great deal of material without removing any of the stromin component. (A quantitative study (13) of the loss of material other than stromin as a function of washing 'stroma' at an acid pH has recently been carried out.) The components which remain associated with the stromin when red cells are hemolysed in glycerol are not known. If a large amount of hemoglobin, S protein, and other material, which may form part of an internal structure of the red cell, remains in the ghost, they probably would be measured by Mitchison's technique and that may account for the large value for the thickness of the ghost which he obtained. We have suggested that stromin, plus the small amount of hemoglobin and S protein intimately associated with it, constitutes the "membrane" of the red cell because the structure of stromin appears to resemble that of a membrane, the components contained in it, *e.g.*, the blood group antigens, are undoubtedly at the surface of the cell, and there is little question that the red cell actually has some form of membrane. It is not possible to resolve at this time the question as to where the membrane ends and the interior structure, if it exists, begins, or if such a demarcation of the two can be made. But on the basis of measuring the thickness of the hemolysed red cell, without taking into account the various components contained in it, the assumption that one is actually measuring the membrane does not appear justified.

SUMMARY

The separation of several apparently distinct fractions from red cell ghosts by means of fractional centrifugation is described. A fraction called stromin is obtained by drying sedimented ghosts, suspended them in water and centrifuging the suspension at an alkaline pH. Another fraction called elinin is obtained in a similar manner after ether extraction of the dried material. A fraction called S protein is present in the supernatant fluids obtained in the above procedures and is separated by precipitation at an acid pH.

The physical and chemical properties of these fractions are described and their possible location in the red cell is discussed. The relationship of these components to those described by other workers is also discussed.

NOTES

1. This work was supported, in part, by grants from the Rockefeller Foundation and the Division of Research Grants and Fellowships of the National Institutes of Health. A portion of it was conducted while the senior author was a Public Health Service Postdoctoral Fellow of the National Heart Institute.

2. This fraction previously was referred to as reticulin, when this work was presented at the Seventh International Congress for Cell Biology, September 8, 1950.

3. The Rh factor was associated with the heavy component of stroma and the clarity of the supernatant fluid proved to be a rough index of the degree of separation. A water clear supernatant fluid invariably contained no Rh factor.

4. This material consisted of hemoglobin, S protein, and perhaps other components. It is referred to as 'colored material' for convenience.

5. This phenomenon has also been referred to as silkiness, ropiness, etc. and is often confused with streaming birefringence. The most complete description of the phenomenon was found in Freundlich's book (p. 403 ff., 11). It is commonly observed in saline suspensions of red cells and suspensions of crystalline materials. It is also observed in water suspensions of ghosts; but whereas this effect is produced by the disk shape of the ghost in this instance, it is produced by the rod shaped particles in the stromin suspensions.

6. Stromin is thought of as being made up of elinin and the ether-extractable lipids, but we do not know if any of the S protein is still associated with it when it is prepared in the manner described. However, the hemoglobin and a large amont of S protein are removed when stromin is prepared in this manner.

7. The length of some of the stromin particles are longer than the diameter of the red cell. This is interpreted as being a result of the manner in which the ghost breaks down under the conditions described. If the membrane surrounds the red cell like the skin on a balloon, a circular strip may be removed from it, like the hoop of a barrel. If this circular strip is cut across its circumference at one spot and then straightened, the strip is longer than the diameter of the circle. This may be the explanation of why the length of some stromin particles exceed the diameter of the red cell. The smaller sized stromin particles may result from the rupture of the larger ones, or by the breakdown of some ghosts into these smaller segments.

8. In making these calculations it is assumed that all the lipids of the red cell are associated with stromin. This assumption may not be valid. Parpart (20) found that all the lipids of the red cell were present in his stroma preparations; Erickson *et al.* (9) reported the recovery of over 90 per cent of the lipids in their stroma preparations; and Waugh and Schmitt (28), on the basis of leptoscope measurements, state that as much as 60 per cent of the lipids of the red cell was lost on hemolysis. In one experiment the supernatant fluid obtained after centrifuging the hemolysate with no pH adjustment was dried and refluxed with alcohol-ether (3 : 1 by volume); approximately ten per cent of the lipids of the red cells were present in the extract. It is possible, however, that the lipids which dissociate under these conditions of hemolysis were associated with stromin, and Parpart's work suggest that this is so.

9. Hunter *et al.* showed (14) that a greater amount of hemoglobin was released when cells were hemolysed at pH 7.2 than at pH 6.3, and suggested that the ghosts were free of hemoglobin at the alkaline pH. The complete separation of hemoglobin from the ghosts at pH 7.2 may occur with chicken cells, which they used, but it does not occur with human cells. They determined the total loss of hemoglobin by observing that the sedimented ghosts in the alkaline solution were "white." However, if there was but a small amount of hemoglobin retained at this pH, and if the ghosts were not tightly packed, they probably would appear "white," especially in contrast to a red supernatant fluid. Their interpretation that the hemoglobin is retained within the ghost at the acid pH may not be correct. It is possible that the hemoglobin co-precipitates with other components of the membrane at the acid pH independent of any permeability process. The fact that some hemoglobin precipitates along with stromin (in suspensions of unextracted stroma) and elinin (in solutions of extracted stroma) at an acid pH (5.5–6.5) suggests that this is the case.

REFERENCES

1. Adams, G. A., *Biochem. J.*, **32**, 646 (1938).
2. Boehm, G., *Biochem. Z.*, **282**, 22 (1935).
3. Bürker, K., *Pflügers Arch. ges. Physiol.*, **195**, 516 (1922).
4. Calvin, M., Evans, R. S., Behrendt, V., and Calvin, G., *Proc. Soc. Exptl. Biol. Med.*, **61**, 416 (1946).
5. Chargaff, E., *Advances in Protein Chem.*, **1**, 1 (1944).
6. Dandliker, W. B., Moskowitz, M., Zimm, B., and Calvin, M., *J. Am. Chem. Soc.*, **72**, 5587 (1950).
7. Dorland, W. A. N., Medical Dictionary, W. B. Saunders Co., Phila., 1942.
8. Drabkin, D., *Science*, **101**, 445 (1945).
9. Erickson, B. N., Williams, H. H., Bernstein, S. S., Arvin, I., Jones, R. L., and Macy, I. G., *J. Biol. Chem.*, **122**, 515 (1938).
10. Evans, R. S., Moskowitz, M., and Calvin, M., *Proc. Soc. Exptl. Biol. Med.*, **73**, 637 (1950).
11. Freundlich, H., Colloid and Capillary Chemistry, New York, 1926.
12. Furchgott, R. F., *Cold Spring Harbor Symposia Quant. Biol.*, **8**, 224 (1940).
13. Howe, C., *J. Immunol.*, **66**, 9 (1951).
14. Hunter, F. R., Stringer, L. D., and Weiss, H. D., *J. Cellular Comp. Physiol.*, **16**, 123 (1940).
15. Jorpes, E., *Biochem. J.*, **26**, 1488 (1932).
16. Keilin, D., and Hartree, E. F., *Nature*, **148**, 75 (1941).
17. Mitchison, J. N., *Ibid.*, **166**, 347 (1950).
18. Moskowitz, M., Dissertation, University of California, Berkeley, 1949.
19. Moskowitz, M., Dandliker, W. B., Calvin, M., and Evans, R. S., *J. Immunol.*, **65**, 383 (1950).
20. Parpart, A. K., *J. Cellular Comp. Physiol.*, **19**, 248 (1942).
21. Parpart, A. K., and Dziemian, A. J., *Cold Spring Harbor Symposia Quant. Biol.*, **8**, 17 (1940).
22. Ponder, E., *J. Exptl. Biol.*, **18**, 257 (1942).
23. —— Hemolysis and Related Phenomena, New York, 1948.
24. —— *Revue d'Hematologie*, **5**, 580 (1950).
25. Rubenstein, D. L., and Ravikovich, H. M., *Nature*, **158**, 952 (1946).
26. Schmitt, F. O., Bear, R. S., and Ponder, E., *J. Cellular Comp. Physiol.*, **9**, 89 (1936).
27. Stern, K. G., Reiner, M., and Silber, R. H., *J. Biol. Chem.*, **161**, 731 (1945).
28. Waugh, D. F., and Schmitt, F. O., *Cold Spring Harbor Symposia Quant. Biology*, **8**, 233 (1940).
29. Webster's Collegiate Dictionary, G. & C. Merriam Co., Springfield, 1939.
30. Wolpers, C., *Naturwissenschaften*, **286**, 461 (1941).

STUDIES ON MITOCHONDRIA

JOHN W. HARMAN and MURIEL FEIGELSON

Department of Pathology, Medical School, University of Wisconsin, Madison

Received April 23, 1951

III. THE RELATIONSHIP OF STRUCTURE AND FUNCTION OF MITOCHONDRIA FROM HEART MUSCLE[1]

Several groups of workers have indicated the importance of integrated (or multi-enzyme) enzyme systems in the processes of cellular respiration (3, 18, 21). One such complex of enzymes has been designated the "cyclophorase" system because it specifically implements the citric acid cycle (4). This and other analogous systems concerned with oxidation and oxidative phosphorylation are associated predominantly with the mitochondria (5, 12, 14, 20). In all tissues from which mitochondria have been separated the dependence of the complex of oxidative enzymes upon mitochondria is manifest.

Several of the earlier studies suggested that the oxidative activity of mitochondria is not only dependent upon their content of the oxidative enzymes but is also significantly influenced by the physical state of the particles (9, 10). A close correlation between the structure of mitochondria and enzymatic activity was subsequently observed (6, 8, 15). Because of this interrelationship of enzyme activity and structural integrity the present study was oriented to elucidate more precisely the mechanism by which the morphology and function of mitochondria are mutually adapted.

EXPERIMENTAL METHODS

1. *Preparation of mitochondria:* A rabbit (2 to 3 kilograms in weight) was stunned by a blow on the head and rapidly exsanguinated by cutting the neck vessels. The heart was removed and immersed in iced water. After rapid weighing it was coarsely minced with a scissors into 10 volumes of ice cold fluid and homogenized in a pre-cooled stainless steel Waring blendor (150 ml capacity) for 75 seconds. The medium was usually 0.25 molar sucrose, containing 8 ml of 0.04 molar sodium bicarbonate per liter so that the final homogenate was about pH 7.0 (17). As experimental variations 0.31 molar and 0.50 molar sucrose solutions were used.

[1] This investigation was supported by a grant from the Life Insurance Medical Research Fund.

The homogenate was strained through 2 folds of surgical gauze (type II, 32 × 28, mesh) and centrifuged for 10 minutes with a force of 150 × gravity.[1] This speed was empirically obtained and found to give reproducible preparations as determined microscopically. The residue of this preparation, consisting of nuclei, myofibrils and incompletely ruptured cells as well as numerous mitochondria, was discarded. The supernate was carefully decanted and centrifuged at the instrument's maximum speed, 2 700 × gravity. During the various centrifugations the suspensions were maintained at 0° C by replaceable ice jackets, which were adequate for each period of centrifugation (22). The supernate was discarded and the residue contained the mitochondria, which were examined at this point for purity with the phase microscope as in previous studies (5). The tightly packed residue was resuspended in 10 volumes of either a sucrose or an electrolyte medium, recentrifuged at 2700 × gravity for 10 minutes, resuspended in a small volume of the same sucrose or electrolyte medium and made up to a final volume equivalent to that of the original volume of cardiac tissue. In several experiments the supernate obtained from the initial slow centrifugation was divided into several equal volumes which were subsequently centrifuged, washed and resuspended separately, using several different types of wash media and suspending fluids. In each step of the preparation, pre-cooled glassware and solutions were used so that the mitochondria were kept at a temperature between 0° C to 4° C at all stages of preparation.

2. *Manometric procedures:* The suspensions of mitochondria were examined for their ability to catalyze the oxidation of citric acid cycle intermediates with the estimation of oxygen uptake, employing conventional Warburg manometric techniques.

The following additions were regularly made to the Warburg flasks, at levels found to be optimal from both the morphological and enzymatic point of view: α-ketoglutarate or other citric acid cycle substrate, $MgCl_2$, adenosine-5-phosphate, and succinate in the main chamber of all flasks. The small quantity (0.5 micromoles per ml) of succinate was added to all flasks in order to maintain enzyme activity until α-ketoglutarate or other substrate was tipped in from the side arm at the end of the equilibration period (2, 4). Thus, a "blank" will refer to a flask containing this amount of succinate in the main chamber but no substrate in the side arm; an "experimental" will refer to a flask containing the succinate in the main chamber and substrate in the side arm. A pH of 7.25 was maintained with a phosphate buffer. The tonicity of the original mitochondrial preparation was maintained in each Warburg flask by adding to the flask an adequate quantity of a more concentrated solution of the suspending fluid (*e.g.*, sucrose, KCl, NaCl). This was an important consideration since studies on osmotic pressure required the same tonicities throughout the duration of the oxidation period. The final volume in the flasks was made up to 2 ml. A CO_2 absorbent (5N NaOH) was used to saturate the filter paper in the center well. After the flasks were set in chipped ice the mitochondrial suspension

[1] A Sorvall angle head centrifuge (Type SP/X) was used. For all gravity manipulations an angle head of 24 cm diameter was employed. The centrifugal forces designated indicate those at the distal extremities of the tubes employed. Due to increased viscosity, when 0.50 molar sucrose was used as the homogenizing fluid, 15 minutes of centrifugation were necessary to produce a comparable degree of sedimentation as was obtained with 0.25 molar or 0.31 molar sucrose in 10 minutes of centrifugation.

was added, oxygen introduced as the gaseous phase, and the flasks thermally equilibrated for 8 minutes in a 37° C water bath. Then the stopcocks were closed, the substrate tipped in from the side arm, and the oxygen uptake measured.

In experiments where the homogenate was subdivided and exposed to various media and suspending fluids, the rates of oxidation were compared quantitatively by an estimation of the quantity of tryptophane, tyrosine and nucleotide as a measure of protein content in each (13). This was accomplished by adding 0.05 or 0.10 ml aliquots of the mitochondrial suspension to 2 ml of 30 per cent KOH, placing the tubes in a boiling water bath for 30 minutes and diluting to 6 ml with water. The absorption by the resultant sample, now only insignificantly turbid, was read in a Beckman spectrophotometer (Model DU.) at a wave length of 280 mμ against a blank containing 10 per cent KOH. One protein unit (P.U.) was arbitrarily defined as that quantity of protein which has an absorption value (log $^{I_0}/_{I}$) of 1.0 by the method herein described. The oxygen uptake was reported as the microliters of oxygen consumed per protein unit (P.U.) during the oxidation period, as indicated for each experiment. Comparisons were made only among the values obtained within a single experiment, since the duration of the oxidation period was varied from experiment to experiment, and the potency of preparations differed from one animal to another.

3. *Cytological technique:* Simultaneously with the introduction of the suspension into the Warburg flask the mitochondrial suspension was examined by phase contrast[1] in the manner de scribed previously (6) and a differential count made of the various forms of mitochondria. When the rate of oxidation began to fall off (usually 40 to 60 minutes) the flasks were removed in rotation and samples studied cytologically. Differential counts were repeated on all flask contents at the end of the period of oxidation. Approximately 250 to 300 mitochondria were usually counted, using the Dark-M contrast which allowed clearest definition. In some instances as few as 150 mitochondria only were counted because the forms altered rapidly in the slide preparation due to the lack of oxygen, temperature changes and absence of substrate. It was preferable, under such conditions, to take several fresh smears during a differential count to obviate spurious variations due to these interfering conditions. The occurrence of clumping sometimes interfered with clearcut counts but was not a serious difficulty. In all experiments the differential counts were made on both "experimental" and "blank" flasks and the data of the "experimental" flasks only was presented in table form, although the information obtained from the "blanks" required evaluation in the results.

The differential counts were made in such a manner that subjectivity was minimized; the identity of the suspension under examination was concealed from the microscopist until the completion of the experiment.

In presentation of the data, single experiments were cited to exemplify the results obtained, although at least 3 experiments had been performed in the elucidation of the particular phenomenon reported.

---

[1] For light microscopy the Spencer Microscope R-4-L was used throughout, and all photographs were taken with use of a Bausch and Lomb Hyperplane (10 ×) ocular. Phase microscopy was carried out with a Spencer Phase system which included a turret condenser and 1.8 mm lens for Dark-M, Light-M and B-Minus contrasts.

RESULTS

1. *Cytological examination of types of mitochondria:* The mitochondria freshly isolated in 0.50 and 0.80 molar buffered sucrose are elongated, beaded rodlets which have different mensurations in their 3 dimensions (Fig. 1). On one side of their flat surface, the more concave aspect, there is usually a clear, white, refractile, oval area. In more dilute sucrose (0.25 and 0.31 molar) the mitochondria swell and become spherical, remaining predominantly dense and homogeneous (Fig. 2). A small, though regularly constant, proportion are differentiated into a central, white "target" refractile area, with a surrounding peripheral dense zone (Fig. 3). These "spherical-dense" and "target" forms are interspersed with a few persistent rods (these 3 types are grouped in tables under the category of "total dense forms"). With further dilution of the medium, the mitochondria are more swollen and become altered internally, either into a hemispherically segmented light and dark zone called a "limbus," or into a dense marginal eccentric "crescent" with a large, clear zone (Figs. 4 and 5). The proportion of these "crescent" and "limbus" forms is an indication of the molar concentration of the medium, although aging initiates their appearance also. Intermediate varieties of these types are seen, but further subdivision is not pertinent to the present study. Variation in the size of the "spherical-dense" mitochondria is considerable, because many minute spherical particles are formed by fragmentation of the beaded and segmented rods into smaller units upon aging the preparation or diluting the suspending medium. For this reason, counts of the absolute number of mitochondria, unless made on the freshly isolated rods, may be open to considerable error and consequently were not determined in this study.

2. *Relationship of type of mitochondria to oxidation:* In the determination of optimal conditions for oxidation of various intermediates of the citric acid cycle, it is found that the requirements for magnesium are especially high (Fig. 6). It is observed that maximum oxidation is attained at a magnesium level of 10 μ moles per ml and is associated with a high concentration of total dense forms and "target" type mitochondria. On the contrary, in the flasks containing low concentrations of magnesium, "crescents" are the prevalent form. The addition of such co-factors as cytochrome-c, co-carboxylase, and diphosphopyridine nucleotide is without significant effect on either the oxidation or the morphology of mitochondria prepared in sucrose.

Using the optimum level of magnesium in the Warburg vessels, suspensions prepared in various concentrations of sucrose were examined cytologically

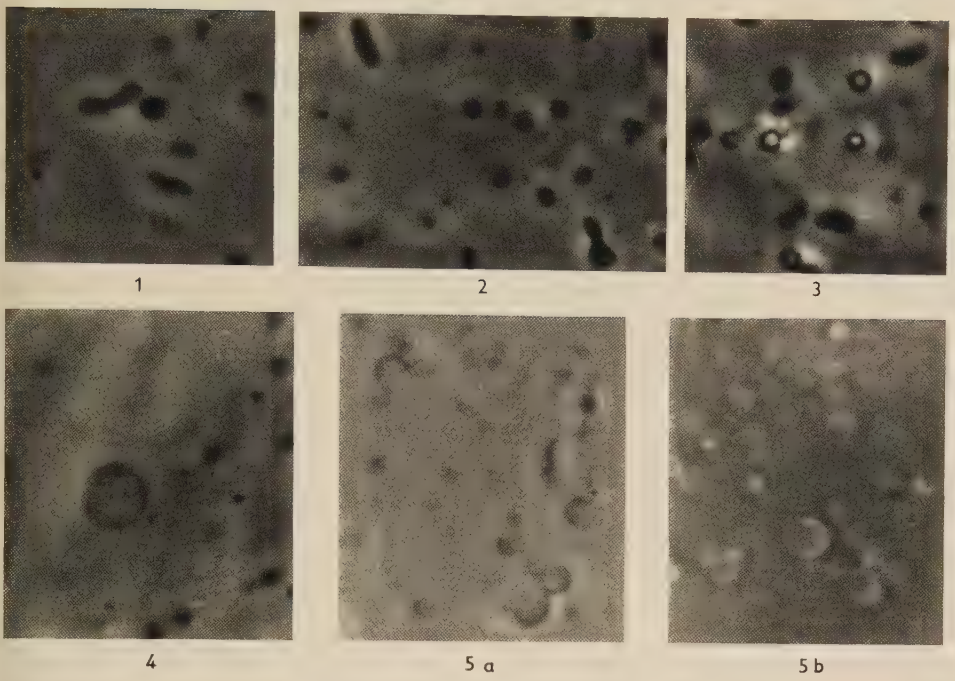


Fig. 1. Several free mitochondria are seen. The rod shape and irregular nodularity are evident in two; the remainder are approximately spherical. Suspending medium is 0.50 M sucrose. Phase Contrast. Dark-M. 2 200 ×.

Fig. 2. The mitochondria are predominantly dense, small spheres. A few are elongated and irregular. Suspending medium is 0.25 M sucrose. Phase Contrast Dark-M. 2 200 ×.

Fig. 3. Many enspherulated mitochondria are differentiated into central light and peripheral opaque zones, assuming a "target" appearance. Alteration of focus does not obliterate the differentiation, as might be expected in surface phenomena. A few dense spheres and rod shaped mitochondria are also observed. The medium is 0.25 M sucrose. Phase Contrast. Dark-M. 2 200 ×.

Fig. 4. A single, large, spherical mitochondrion occupies the center of the field. It is divided into light and dense hemispheres and is designated "limbus" mitochondrion. The dense area represents the residuum of the original dense body from which the lighter excrescence emerges. Suspending medium is 0.25 M sucrose, diluted by several volumes of distilled water. Phase Contrast. B-Minus. 2 500 ×.

Fig. 5 A. The mitochondria are enspherulated and enlarged (Compare Fig. 2 and 3). Several are "limbus" forms; the remainder have a narrow eccentric density which occupies a segment of the periphery. This latter form is the "crescent" type. Suspending medium is 0.25 M sucrose, replaced by diffusion of distilled water under the cover slip. Phase Contrast. Dark-M. 2 200 ×.

Fig. 5 B. The conditions of examination are identical with those for Fig. 5 A, using the same suspension of mitochondria. Most mitochondria contain the eccentric, dense, narrow zones. Phase Contrast. Bright-M. 2 200 ×.

before and after the oxidation period. It is noticed in Table I that the initial form of the mitochondria has no significant influence on either their oxidative capacity or the final shape. The suspension in 0.50 molar sucrose com-

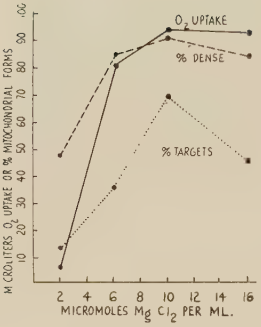


Fig. 6. The effect of magnesium on the oxidation and structure of mitochondria. The heart was homogenized and the mitochondria suspended in 0.25 M sucrose. 0.4 ml of mitochondrial suspension was added to the Warburg flasks. The flasks were made up to a final volume of 2 ml and contained per ml of fluid the following additions: 0.5 μ moles of sodium succinate; 1.5 μ moles of adenosine-5-phosphate (Schwarz Laboratories, Inc.); 15 μ moles of potassium phosphate buffer, pH 7.25; 3 μ moles of sodium α-ketoglutarate (added to the side arm of "experimental" flasks); and magnesium chloride at the levels indicated. A concentrated solution of the suspending medium was added so that the final molarity in the flask was equal to that in the original mitochondrial suspension (i.e., 0.50 M sucrose was added to obtain a final concentration of 0.25 M sucrose). The filter paper in the center well was saturated with 0.2 ml of 5 N NaOH. The duration of oxidation period was 41 minutes.[1]

mences with a high concentration of rods and "targets," oxidizes slowly, fails rapidly and ends as a suspension of "crescents" mainly; whereas the suspensions in 0.25 molar sucrose are predominantly made up of "spherical-dense" mitochondria which, after a considerable oxygen uptake, finally contained "target" mitochondria in relative abundance. Suspensions in 0.31 molar sucrose hold an intermediate position between the hypo- and hypertonic solutions from both a cytological and enzymatic point of view. There is a distinct conversion of "spherical-dense" forms to "targets" in the 0.25 molar sucrose suspension, which is most active oxidatively. Although the exact mechanism by which variations in the sucrose concentration alter the properties of mitochondria is complex, it is evident that there is a close

Table I. The mitochondria obtained by homogenization in 0.50 M sucrose were divided into three equal volumes and were washed and suspended in 0.25 M, 0.31 M and 0.50 M sucrose respectively. 0.5 ml of mitochondrial suspension was added to the Warburg flasks. The flasks were made up to a final volume of 2 ml and contained per ml of fluid the following additions: 0.5 μ moles of sodium succinate; 1.5 μ moles of adenosine-5-phosphate; 15 μ moles of potassium phosphate buffer, pH 7.25; 3 μ moles of sodium α-ketoglutarate (added to the side arm of "experimental" flasks); 10 μ moles of magnesium chloride. A concentrated solution of the suspending medium was added so that the final molarity in the flask was equal to that in the mitochondrial suspension used. The filter paper in the center well was saturated with 0.2 ml of 5 N NaOH. The duration of the oxidation period was 69 minutes.

Table II. The mitochondria obtained by homogenization in 0.25 M sucrose were divided into two equal volumes and were washed and suspended in 0.25 M sucrose and distilled water respectively. Preparation of the Warburg flasks was described in Table I. As indicated in the table, the following additions were made per ml of fluid: 20 γ of cocarboxylase (Nutritional Biochemicals Corp.); 0.02 μ moles of cytochrome-c (Sigma Chemical Co.); 150 γ of diphosphyridine nucleotide (DPN) (Schwarz Laboratories, Inc.); 10 μ moles of nicotinamide (Merck and Co., Inc.); and 0.1 ml of "Kochsaft" (prepared according to Lardy (11)). The duration of the oxidation period was 60 minutes for Experiment A and 42 minutes for Experiment B.

[1] Unless otherwise indicated, *in this figure and all subsequent tables*, "μ liters O_2 uptake" represents the O_2 uptake due to the addition of α-ketoglutarate, "blanks" having been subtracted. Dense forms include spherical, rod and "target" forms of mitochondria.

TABLE I

The effect of sucrose concentration on the oxidation and structure of mitochondria.

Final sucrose concentration	μ liters O_2 uptake per P. U.	Mitochondria before oxidation period (per cent)				Mitochondria after oxidation period (per cent)			
		Total dense forms			"Limbus" and "crescent"	Total dense forms			"Limbus" and "crescent"
		Rods	"Spherical-dense"	"Targets"		Rods	"Spherical-dense"	"Targets"	
0.25 M	64.0	0	68	6	26	0	44	35	21
0.31 M	53.8	6	60	27	7	0	65	12	23
0.50 M	16.1	21	50	22	7	0	47	0	53

TABLE II

The effect of distilled water on the oxidation and structure of mitochondria.

Experiment	Medium	Additions	μ liters O_2 uptake per P. U.	Mitochondria before oxidation period (per cent)			Mitochondria after oxidation period (per cent)			
				Total dense forms		"Limbus" and "crescent"	Total dense forms		"Limbus"	"Crescent"
				"Spherical-dense"	"Targets"		"Spherical-dense"	"Targets"		
A	0.25 M sucrose	none	83.8	86	8	6	33	57	10	
	distilled water	none	23.8	5	±	95	4	0	96	
	distilled water	cocarboxylase cytochrome-c DPN nicotinamide	52.3	5	±	95	6	0	94	
B	0.25 M sucrose	none	53.3	85	10	5	49	47	4	
	0.25 M sucrose	"Kochsaft"	57.7	85	10	5	32	65	3	
	distilled water	none	22.2	4	0	96	0	0	1	99
	distilled water	"Kochsaft"	38.6	4	0	96	0	0	14	86

TABLE III

The effect of substrate on the oxidation and structure of mitochondria.

Flask	μ liters O_2 uptake	Mitochondria after oxidation period (per cent)		
		Total dense forms		"Limbus" and "crescent"
		"Spherical-dense"	"Targets"	
"Blank"	34	64	0	36
"Experimental"	228[1]	36	55	9

TABLE III. The heart was homogenized, and the mitochondria washed and suspended in 0.25 M sucrose. Components of the Warburg flasks were as in Table I. The duration of the oxidation period was 60 minutes.

correlation of "spherical-dense" and "target" forms at the end of the oxidation period, with high oxygen uptake. On the contrary, exposure of the suspension to distilled water converts nearly all the mitochondria into "crescents" and simultaneously depresses oxidation (Table II). However, the addition of co-factors and "Kochsaft" to the suspension of mitochondria in distilled water partially restores oxidation to the level of the preparation in 0.25 molar sucrose. This partial restoration of oxidation is accompanied by the appearance of "limbus" mitochondria in the suspension.

In view of the occurrence of "crescents" in the flasks exhibiting poor oxidation, the effect of substrate was also noted (Table III). In the "blank" flask the presence of the minute quantity of succinate does not preclude the appearance of "crescents", whereas the addition of 2 μ moles of α-ketoglutarate per ml prevents their development, in correlation with the con-

TABLE IV. The heart was homogenized and the mitochondria washed and suspended in 0.25 M sucrose. 0.5 ml of mitochondrial suspension was added to the Warburg flasks. The flasks were made up to a final volume of 2 ml and contained per ml of fluid the following additions: 3 μ moles of sodium α-ketoglutarate (added to the side arm); 10 μ moles of sodium citrate where indicated; 1.5 μ moles of adenosine-5-phosphate; 15 μ moles of potassium phosphate buffer, pH 7.25; and 10 μ moles of magnesium chloride. The final concentration of sucrose was adjusted with 0.50 M sucrose to 0.25 M. The filter paper in the center well was saturated with 0.2 ml of 5 N NaOH. After 30 minutes in the Warburg bath flasks 3 and 4 were tipped; flasks 1 and 2 were removed for cytological examination.

TABLE V. In each experiment the mitochondria obtained by homogenization in 0.50 M sucrose were equally divided and were washed and suspended in the media indicated. Components of the Warburg flasks were as in Table I. The duration of the oxidation period was 40, 45 and 50 minutes for Experiments A, B and C respectively.

[1] The "blank" value was not substracted, so that comparisons between "blank" and "experimental" values could be made.

Flask	Citrate	μ liters O_2 uptake in 30 min.[1]	Mitochondria after 30 minutes (per cent)			α-keto-glutarate tipped in at 30 min.	μ liters O_2 uptake in 48 min.[1]	Mitochondria after 48 minutes (per cent)		
			Total dense forms		"Limbus" and "crescent"			Total dense forms		"Limbus" and "crescent"
			"Spherical-dense'	"Targets"				"Spherical-dense"	"Targets"	
1	—	7.6	41	0	59	—	—	—	—	—
2	+	26.4	73	20	7	—	—	—	—	—
3	—	7.1	—	—	—	+	14.2	57	±	43
4	+	20.8	—	—	—	+	77.4	58	38	4

[1] μ liters O_2 uptake were not corrected for "blanks."

TABLE V

The effect of electrolytes on the oxidation and structure of mitochondria.

Experiment	Molarity of medium			μ liters O_2 uptake per P. U.	Mitochondria before oxidation period (per cent)				Mitochondria after oxidation period (per cent)			
					Total dense forms			"Limbus" and "crescent"	Total dense forms			"Limbus" and "crescent"
	Sucrose	KCl	NaCl		Rods	"Spherical-dense"	"Targets"		Rods	"Spherical-dense"	"Targets"	
	0.31	—	—	54.5	29	50	19	2	0	48	36	16
A	—	0.15	—	31.9	0	45	13	42	0	58	16	26
	—	—	0.15	8.4	0	70	11	19	0	67	17	16
	—	0.06	—	86.0	0	30	0	70	0	46	0	54
B	—	0.12	—	68.7	0	64	6	30	0	76	14	10
	—	0.20	—	31.0	0	72	0	28	0	69	15	16
	0.25	—	—	29.7	0	90	±	10	0	48	18	34
C	0.25	0.125[1]	—	9.7	0	60	±	10	0	63	19	18
	0.50	—	—	6.8	50	49	±	1	0	66	±	34

[1] The mitochondria which were washed and suspended in 0.25 M sucrose were used. In the flask, however, the tonicity was made iso-osmotic with 0.50 M sucrose by the addition of KCl to obtain a concentration of 0.125 M, and sucrose to obtain a concentration of 0.25 M.

siderable oxygen uptake. Other substrates including succinate, malate and citrate produce similar cytological effects, although citrate is poorly oxidized compared with the others.

It appears that a high oxidation level is not necessarily required to maintain the "spherical-dense" and "target" types of mitochondria, but that even a minimal level of oxidation is able to maintain these forms. An experiment was oriented to test this further (Table IV). Since it was seen that citrate is slowly oxidized, it was used as substrate for 30 minutes to sustain the suspension. At that time α-ketoglutarate was tipped in from the side arm. A rapid burst of oxidation occurs and the suspension contains a significantly high concentration of "target" mitochondria. It was observed that the addition of α-ketoglutarate without preconditioning with citrate allows little further increase in oxidation and that the mitochondria are mainly "crescent" and "spherical-dense" without "targets." The minimal sustaining oxidation of citrate, therefore, is associated with the maintenance of "target" mitochondria.

The preparation and suspension of the mitochondria in iso-osmotic potassium chloride depresses oxidation below the levels found in iso-osmotic sucrose suspensions (Table V, Experiment A). With this low level of oxidation it is noticeable that the suspensions at the end of oxidation contain few "targets," but are composed mainly of dense spheres and "crescents" which have usually oxidized less actively. The presence of the "spherical-dense" mitochondria does not give maximal oxygen uptake, although they indicate a substantially greater oxidation than is associated with "crescents." Variation in the concentration of potassium chloride (Table V, Experiment B) affects oxidation in a manner comparable to that seen with variation of sucrose concentration (Table I), although after the oxidation period similar change in form is not evident: the high concentration of potassium has enabled dense spheres to predominate, whereas in high sucrose concentrations there are few dense forms. In a mixture of sucrose and potassium chloride which approximates the tonicity of 0.50 molar sucrose (Table V, Experiment C), oxidation is depressed to a level comparable to that of the pure sucrose solution. The population of mitochondria in the potassium chloride-sucrose medium, however, is mainly dense forms with a high concentration of "target" forms, whereas in the 0.50 molar sucrose preparation, "targets" are virtually absent. The presence of the electrolyte is not deleterious to shape, even in hypertonic solution; on the other hand, the rate of oxidation is somewhat depressed by it.

The use of sodium chloride as the electrolyte depresses oxidation still

further (Table V, Experiment A), although the differential mitochondrial composition of the suspension is approximately the same as that seen in an iso-osmotic solution of the potassium salt. In the "blank" suspensions in potassium and sodium chloride the proportion of "spherical-dense" mitochondria is much higher than in iso-osmotic sucrose, suggesting again an effect of saline alone in the conversion and maintenance of these forms.

DISCUSSION

The earlier studies on oxidation by mitochondria established that the maintenance of the cyclophorase complex and the integrity of the structure of mitochondria were interdependent (6). The obliteration of the internal structure of the mitochondria accompanied cessation of cyclical oxidation, but the relationship appears to be even more precise and positive because several states of the mitochondria affect and appear to govern the rate of oxidation.

The rod forms, which are the usual intracytoplasmic shape in heart muscle (7), allow only slow oxidation. Although this may appear a paradox, it is not out of place to believe that such a limited rate of oxidation may be more appropriate to the regulated events of the intracellular environment, and that such a dampened effect is also attributable to the high intracellular potassium levels. The observations of Riley (19) indicate that such a salt effect on cellular oxidation is probable. At the opposite extreme the isolated, swollen, crescent-shaped mitochondria have a diminished capacity to metabolize. It is interesting that this is the form which characterizes the peculiar cellular alteration called "cloudy swelling" (1, 16, 23) which is a type of degeneration well known to pathologists.

Between these extreme variations there are numerous transitional forms. It is probable that fluctuations in shape and size of the mitochondria, which are amply described in the older literature, may reflect the sequence of metabolic events in the cells and contribute by their metamorphoses to metabolic regulation.

SUMMARY

The shape of the mitochondria from heart muscle is correlated with oxidative activity. Both osmotic pressure and the concentration of magnesium exert a comparable influence on morphology and oxidation. On the other hand, several electrolytes depress oxidation without alteration of mitochondrial structure.

The authors wish to express their thanks to Professor D. Murray Angevine for his constant interest, to Professor Van R. Potter for his advice, and to Mr. Homer Montague for his unfailing assistance with the photographic procedures.

REFERENCES

1. Anitschow, N., *Verhandl. deut. path. Ges.*, **17**, 103 (1914).
2. Cohen, P. P., and Hayano, M., *J. Biol. Chem.*, **166**, 251 (1946).
3. Dixon, M., Multi-Enzyme Systems. Cambridge University Press, London, p. 13, 1949.
4. Green, D. E., Loomis, W. F., and Auerbach, V. H., *J. Biol. Chem.*, **172**, 389 (1948).
5. Harman, J. W., *Exptl. Cell. Res.*, **1**, 382 (1950).
6. —— *ibid.*, **1**, 394 (1950).
7. —— *ibid.*, **3**, 58 (1952).
8. Hogeboom, G. M., and Schneider, W. C., *Nature*, **166**, 302 (1950).
9. Hogeboom, G. M., Schneider, W. C., and Pallade, G. E., *J. Biol. Chem.*, **172**, 619 (1948).
10. Kennedy, E. P., and Lehninger, A. L., *ibid.*, **179**, 957 (1949).
11. Lardy, H. A., in Umbreit, W. W., *et al.*, Manometric Techniques and Tissue Metabolism, Burgess Publishing Co., Minneapolis, p. 218, 1949.
12. Lehninger, A. L., and Kennedy, E. P., *J. Biol. Chem.*, **173**, 753 (1948).
13. Lerner, A. B., and Barnum, C. P., *Arch. Biochem.*, **10**, 417 (1946).
14. Leuthart, F., and Mauron, J., *Helv. Physiol. Pharmacol. Acta*, **8**, 386 (1950).
15. MacFarlane, M. G., *Biochem. J.*, **47**, XXIX (1950).
16. Opie, E. L., *J. Exptl. Med.*, **86**, 45 (1947).
17. Potter, V. R., in Umbreit, W. W., *et al.*, Manometric Techniques and Tissue Metabolism, Burgess Publishing Co., Minneapolis, p. 137, 1949.
18. Potter, V. R., LePage, G. A., and Klug, H. L., *J. Biol. Chem.*, **175**, 619 (1948).
19. Riley, V., *Proc. Soc. Exptl. Biol. Med.*, **75**, 644 (1950).
20. Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, **177**, 893 (1949).
21. Stern, K. G., *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 318 (1939).
22. Stocking, C. R., and Weier, T. E., *Science*, **111**, 520 (1950).
23. Zollinger, H., *Schweiz. Z. allgem. Path. u. Bakt.*, **11**, 617 (1948).

IV. THE CYTOLOGICAL LOCALIZATION OF MITOCHONDRIA IN HEART MUSCLE[1]

In preceding publications (7, 8) evidence was assembled to show that the mitochondria in liver, kidney and myocardial cells are the cellular components which contain a complex of oxidative enzymes designated as "the cyclophorase system" (6). Subsequent investigations (9) indicated that the oxidative capacity of the mitochondria from heart muscle is intimately affected by their form and that there is a close interrelationship of structure and metabolic function.

[1] This investigation was supported by a grant from the Life Insurance Medical Research Fund.

In view of this interdependence of shape and oxidative activity, the precise position and shape of the intracellular mitochondria assume considerable importance. Many granules have been described in heart and various skeletal muscles (11) commencing with the first descriptions by Kölliker (16, 17), who termed them "interstitial granules," which were later also called sarcosomes by Ebner (19). Some observers distinguished between "A" granules and "I" granules (4, 12, 13) because of a respective tendency of the granules to take up position adjacent to either the "A" or "I" segments of myofibrils, but denied their relationship to mitochondria. A few subsequent workers suggested a relationship to mitochondria (1, 2) although there was considerable confusion with the liposomes, which frequently occur in cardiac and skeletal muscle.

A more detailed study of the metamorphoses of the cardiac mitochondria and their orientation to other intracellular structures was undertaken to define their connection with the granules described by these previous investigators. The various morphological states of the mitochondria were examined in relation to the intracellular topography, the cellular functions and the alteration of the cell morphology with pathologic states.

METHODS

The animals used were albino rabbits, male, weighing 2 500 to 3 000 gms, and male Sprague-Dawley rats weighing 250 gms. All were fed a staple diet (Purina chow) and were not intentionally fasted prior to use.

Homogenates of rabbit heart were prepared in the manner previously described (9). The residue was used for the study of intracellular mitochondria. It was resuspended at 0° C in an equal volume of either sucrose or saline solution. Smears of this were made on glass slides and fixed in sealed Coplin jars for 6 hours over the vapors of 4 per cent osmium tetroxide. These were stained with Aniline Fast Green FCF (10). Wet smears of the resuspended residues were examined by phase contrast microscope as hitherto reported (7). Because of the tendency of the mitochondria to deteriorate and alter shape rapidly at room temperature, especially in the absence of substrate (9), repeated fresh smears were examined to ensure the study of typical normal forms. This degenerative change is peculiarly rapid in suspensions made in the electrolyte solutions.

The mitochondria were stained supravitally by both Janus Green B and Pinacyanole. Glass slides previously flooded with 0.25 ml of 1/50 000 alcoholic solution of either dye were dried by uniform evaporation and used for the smears. On such slides the mitochondria may rapidly acquire the stain and concentrate it selectively. For this type of identification the light microscope was not replaceable by phase contrast.

RESULTS

1. *Demonstration of mitochondria in heart.* The mitochondria freed from the ruptured cells and dispersed into the 0.88 molar and 0.45 molar sucrose solutions are elongated rods which vary in thickness throughout their length and have several areas of constriction at irregular intervals (Fig. 1). In the evolutions due to Brownian movement it is discerned that they are flattened in one dimension and slightly concavo-convex on the flatter surface. Within the concave surface an oval refractile area frequently represents the bead-like bodies separated by the described constrictions. Such mitochondria are tightly packed between and around the myofibrils in the unruptured and partly disintegrated muscle cells which constitute the bulk of the residue.

The intracellular mitochondria are arranged both transversely to and parallel to the long axis of the myofibrils. The units in parallel position (Fig. 2) stretch along several segments of the myofibrils, spanning two or even more sarcomeres in their length. No delimitation is seen in relation to either isotropic "I" segment or to the anisotropic "A" segment of the sarcomere; neither the sarcomere nor the membrane of Krause which defines it are exclusively related to the areas of constriction and beading along the mitochondria. It is clear that the localization of the parallel mitochondria is random with regard to the sarcomeres, although they are situated nearly end to end and completely occupy the interval between the myofibrils. As seen in the homogenates, there is no evidence that they are related to stages of relaxation or contraction. Nor is it readily apparent that the irregularities of the mitochondria are impressed upon them by the distortions of form in the contraction of sarcomeres, since: 1) they lack regular apposition to the sarcome; and 2) the free mitochondria maintain their shapes and are not easily altered or deformed mechanically.

On the contrary, the transverse mitochondria occupy an exact position. They lie across the A-segment of the sarcomere and may straddle two myofibrils (Fig. 3). Most transverse mitochondria appear bent over the myofibril's cylindrical surface; by altering the depth of focus this relationship is easily observed. Wherever these mitochondria are clearly defined they are invariably in contact with the anisotropic segment, so that between adjacent mitochondria the Z-lines and isotropic segment are visible. No separate granules are seen in either of the segments in fresh preparations suspended in 0.88 and 0.45 molar sucrose.

2. *Transformation of mitochondria into granules.* When the homogenates

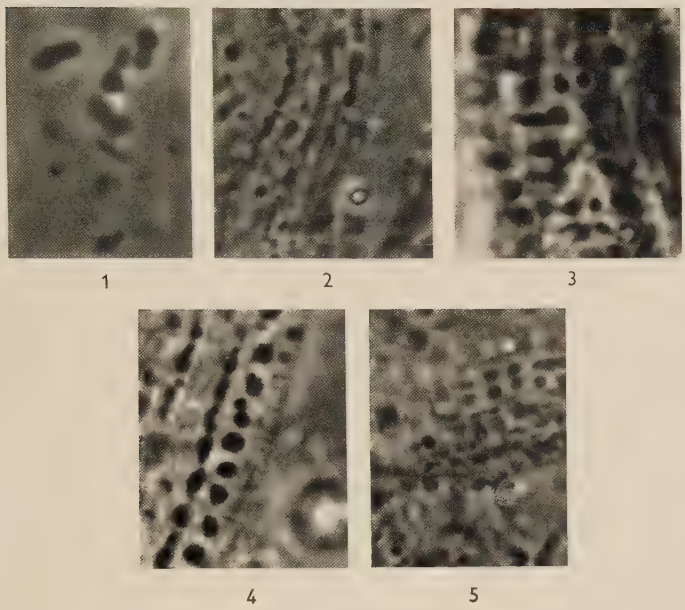


Fig. 1. Several free floating mitochondria, elongated and beaded in shape, are seen. Preparation suspended in 0.88 M sucrose. Some such mitochondria on rotation have areas of different refractivity in their structure. Phase Contrast. Dark-M. 2 200 ×.

Fig. 2. The elongated mitochondria lie parallel to and between the transversely striated myofibrils. A few are enspherulated. One free mitochondrion is a "target" form, with central light refractile area. Phase Contrast. Dark-M. 2 200 ×.

Fig. 3. Several elongated mitochondria are irregular in contour and arranged transversely to the myofibrils. Suspension made in 0.88 M sucrose. Phase Contrast. Dark-M. 2 200 ×.

Fig. 4. The mitochondria in 0.25 M sucrose have assumed spherical shape and are seen overlying the myofibrils. Change of shape from rods was accelerated by dilution of the medium with several volumes of distilled water. Phase Contrast. Dark-M. 2 200 ×.

Fig. 5. Many of the spherical mitochondria lie exactly against the anistotropic discs of the myofibrils, the position of "A" granules. The suspension is in 0.25 M sucrose. Phase Contrast. Dark-M. 2 000 × Magnification.

are prepared and suspended in either 0.25 molar or 0.125 molar sucrose the mitochondria assume a spherical shape (Fig. 4). They are mainly dense and homogeneous, although a small proportion take on a configuration which resembles a "target." The spheres are derived either from entire mitochondria when they are relatively large, or by fragmentation of the mitochondria into their several beads which represent the smaller units. Both these events are seen in suspensions when dilute fluid is allowed to diffuse under the cover slip during the period of observation. The transformation is usually very rapid for any particular mitochondria, although a population change extends over a considerably longer interval.

The spheres which replace the transverse mitochondria are situated in the middle of the sarcomere against the "A" segment. Even the especially large forms are limited by the Z-lines. The precision of the relationship to the anisotropic segment is clearly depicted in Figure 5. This regular relationship is the morphological observation which induced certain observers to use the term "A" granules for such altered mitochondria.

The parallel, interfibrillary mitochondria may round up or disintegrate into separate spheres of varying diameter. When several occur, one or more may lie adjacent to or over an isotropic "I" segment so that the sarcomere is crowded with "I" granules as well as "A" granules. The random distribution of the original rods causes a similar arrangement of the spheres and the absence of a regular arrangement along the sarcomeres. In another respect a certain regularity is missing, insofar as the spheres are sometimes large, other times tiny in relation to both segments, whereas the "I" and "A" granules are described as respecticely small and large by their originators.

3. *Effect of electrolytes on mitochondria.* The use of 0.25 and 0.125 molar potassium chloride for the suspending fluid permits the appearance of forms described elsewhere (8) as crescents. Their location in the cell indicates that, as believed hitherto, they arise by further metamorphosis of the spherical mitochondria. Their relation to the sarcomere is exactly the same as the mitochondria from which they arise. When seen in large numbers they obscure the details of the cell, give it a foamy appearance and constitute the dominant cytological element.

The crescentic, swollen mitochondria, in free state or within the cell, fail to concentrate either Janus Green B or Pinacyanole in quantity adequate for detection. It is undecided whether this is owing to the spreading out of mitochondrial substance by swelling so that the attached dye is not apparent. The fact that certain enzyme reactions are not so active in these forms as in the dense spheres and rods is consistent with this explanation. It may, on the contrary, be because the process of spreading inactivates certain specific reactant groups.

DISCUSSION

It has been possible to establish the metamorphosis of myocardial granules from mitochondria. The genesis of such granules ("A" and "I" sarcosomes) is expedited by the dilute media employed by previous investigators. Such media, although isotonic with plasma, are not iso-osmotic with the intracellular environment which sustains the mitochondria (9). There is no proof that the granules as such are normal, whereas some agreement exists that

the rod shape is consistently a normal form *in vivo*. This being so, the schemata (11, 13) elaborated to explain the role of granules in the contractile process are stultified. Moreover, the absence of such granules from many muscles (14, 15), and specifically of the mitochondria, as observed in studies in this laboratory, does not preclude efficient contraction.

Although the granules as such may be displaced from primary consideration in cell physiology, the function of the mitochondria remains to be explained. It is pertinent that the mitochondrial content of skeletal muscles varies widely among different species and that in the same species there are variations among the several muscles. In our laboratory it is observed that the breast muscles of the pigeon and the bat are unusually rich in mitochondria, and in nearly all mammals the diaphragms are similarly rich. All species of hearts examined have an enormous quantity of mitochondria. In all these tissues that contain many mitochondria there is a high ratio of oxidative activity, especially toward intermediates of the citric acid cycle. It is feasible to infer that the basic contractile process of muscle, in which the sarcomere is a remarkably constant structure, is varied in special instances by the coaption of mitochondria and their systems of oxidative enzymes (18) into the phenomena. Most of these highly oxidative muscles are involved in functions vital to the species and may require release from the hazard of the oxygen debt usual to most skeletal muscles.

The manner in which the energy derived from the mitochondrial oxidative process is adapted to the needs of the myofibrils is a special problem. Yet the intimate relation of mitochondria to myofibrils, which maintain a close physical contact, would indicate the transfer of substances useful to the myofibrils. Although evidence is lacking as to the nature of such substances, the coupling of phosphorylation with oxidation in the mitochondria (3, 5) suggests that the formation and transfer of high energy phosphate bonds may be one of the principal functions of mitochondria in heart and skeletal muscle.

SUMMARY

The mitochondria in heart muscle are rod shaped, beaded structures which under certain conditions are converted to granules. They are interfibrillary in position and may be either parallel or transverse to the sarcomere. Only the transverse mitochondria maintain an exact relation to the sarcomere and lie in apposition to the anisotropic segment. The intimate relation to the myofibrils suggests an active contribution to the contractile process.

REFERENCES

1. Bell, E. T., *Intern. Monatschr. Anat. Physiol.*, **28**, 297 (1911).
2. Bullard, H. H., *Amer. J. Anat.*, **19**, 1 (1916).
3. Cross, R. J., Taggart, J. V., Covo, G. A., and Green, D. E., *J. Biol. Chem.*, **177**, 655 (1949).
4. Engelmann, T. W., *Arch. ges. Physiol.*, **18**, 1 (1878).
5. Green, D. E., Atchley, W. A., Nordmann, J., and Teply, L. J., *Arch. Biochem.*, **24**, 359 (1949).
6. Green, D. E., Loomis, W. F., and Auerbach, V. H., *J. Biol. Chem.*, **172**, 389 (1948).
7. Harman, J. W., *Exptl. Cell Res.*, **1**, 382 (1950).
8. —— *ibid.*, **1**, 394 (1950).
9. —— *ibid.*, **3**, 47 (1952).
10. —— *Stain Technol.*, **25**, 69 (1950).
11. Holmgren, E., *Anat. Anz.*, **31**, 609 (1907).
12. —— *Arch. mikroskop. Anat. Entwicklungsmech.*, **75**, 240 (1910).
13. Jordan, H. E., *Amer. J. Anat.*, **27**, 1 (1920).
14. Knoll, Ph., *Sitzber. Akad. Wiss. Wien Math. naturwiss. Klasse*, **98**, 456 (1889).
15. —— *Denkschr. Kais. Akad. Wiss. Wien Math. naturw. Klasse*, **58**, 633 (1891).
16. Kolliker, A., *Z. wiss. Zoöl.*, **8**, 311 (1857).
17. —— *ibid.*, **47**, 689 (1888).
18. Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, **177**, 893 (1949).
19. von Ebner, V., *Sitzber. Akad. Wiss. Wien Math. naturw. Klasse*, **129**, 93 (1920).

THE MICROSTRUCTURE OF THE SPERMATOZOA OF THE SNAIL *VIVIPARUS*

JEAN HANSON, J. T. RANDALL and S. T. BAYLEY

Wheatstone Physics Laboratory and Medical Research Council Biophysics Research Unit, King's College, London, Great Britain

Received May 15, 1951

The structure and development of the spermatozoa of *Viviparus viviparus* (formerly called *Paludina vivipara*) were described in detail by Meves (11, 12), who made most of his observations on sections of the testis. Using similar methods, sometimes supplemented by studies on vitally-stained unfixed material, later workers (1, 2, 3, 5, 10, 13, 21) have added little to the information published by Meves, with the exception that Pollister and Pollister (15) have given a much fuller account of the behaviour of chromosomes and centrioles during the divisions of the spermatocytes. Following work in this laboratory on the microstructure of ram spermatozoa (18), we have re-investigated the spermatids and spermatozoa of *V. viviparus* (L.) with the aid of some of the newer cytological techniques, especially electron microscopy, phase-contrast microscopy, and some cytochemical staining methods.

Like other prosobranch snails *V. viviparus* produces two kinds of spermatozoa which look very different from each other (Text-fig. 1). The eupyrene spermatozoa, which fertilise the eggs, have nuclei containing the full haploid number of chromosomes, namely seven. The oligopyrene spermatozoa, which are at least as numerous as the eupyrene forms, possess only one chromosome. Although they are found in the female reproductive tract after copulation, they are infertile; theories of their function are discussed on p. 76–77.

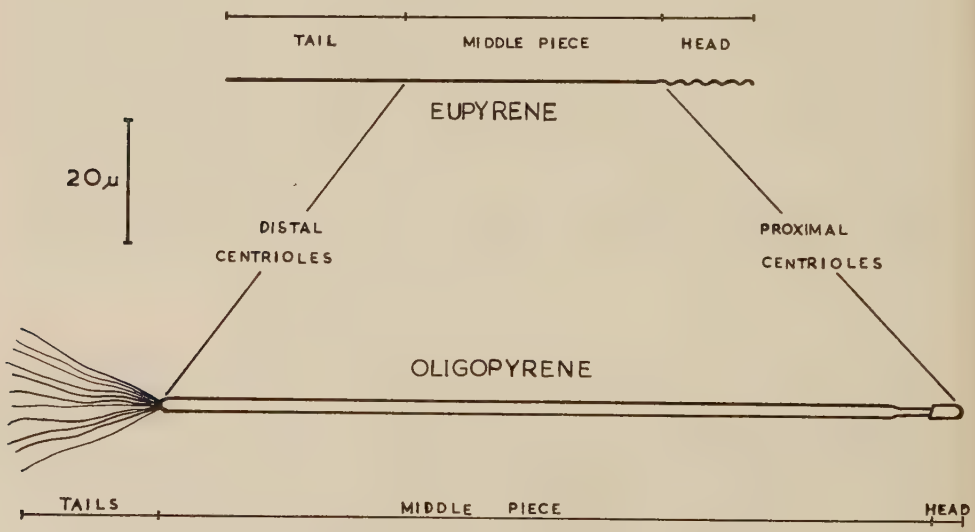
MATERIAL AND METHODS

As little seems to be known about the breeding season of *V. viviparus* in England, it may be useful to record that in snails obtained from ponds in Surrey, mature and developing eupyrene and oligopyrene spermatozoa were found in the testis throughout the year from March 1950 to February 1951. From June 1950 until February 1951 females were examined each month, and motile spermatozoa were always found in some individuals.

Tyrode solution (6) was found to be a suitable medium for keeping living material in a healthy state. Spermatozoa and spermatids from the testis remained motile in Tyrode at room temperature for at least 24 hours, and kept their normal structure. The middle-piece of the oligopyrene forms readily disintegrates in adverse conditions, and its appearance is a convenient index of the normality of a preparation. In Hédon-Fleig solution (6) motility decreased and oligopyrene forms began to disintegrate after about eight hours at room temperature. In preparations contaminated with tissues from other parts of the animal, especially from the digestive gland, similar effects were seen after a shorter period.

Although most of the results reported in this paper were obtained by electron microscopy, their interpretation is based on investigations of living material in the phase-contrast microscope and of fixed and stained smears and sections in the ordinary light microscope. A variety of fixatives and stains was used. Duplicate samples of material studied in the electron microscope were always examined as stained smears in the light microscope.

All specimens prepared for the electron microscope were fixed in 4 per cent formaldehyde, washed in glass-distilled water, placed on formvar-coated grids, dried, and then shadowed with gold-palladium alloy. In order to reveal internal structure, some specimens were partially disintegrated, before fixation, by ultrasonic radiation or in a Potter and Elvehjem homogeniser (17) surrounded by ice. We are grateful to Dr. G. G. Selman for treating material with ultrasonics in the apparatus described by Selman and Wilkins (20). Treatment at 10 W/cm^2 for 2 $\frac{1}{2}$–3 mins. gave satisfactory results. Most of the electron micrographs were taken in a Metropolitan-Vickers E. M. 3 instrument operated at 100 Kv. A few were taken in a Siemens instrument operated at 80–90 Kv., and we are grateful to Dr. R. Bovey and Dr. W. G. P. Lamb who took these micrographs.



Text-fig. 1. Diagram to show structure resolvable in the light microscope.

Text-fig. 2. Head of eupyrene spermatozoon after exposure to a hypertonic medium. Fixed in 4 per cent formaldehyde and stained with iron haematoxylin.

RESULTS

1. *Eupyrene Spermatozoa*

Most of our observations were made on mature spermatozoa from the testis; no differences were found in spermatozoa from fertilised females. The spermatozoon consists of a spirally-twisted head, a long middle-piece and a tail (Text-fig. 1). A proximal centriole is situated between the head and the middle-piece, and a distal centriole between the middle-piece and the tail.

In the electron microscope intact heads are opaque to electrons of the velocity used, and show no surface markings of special interest. A superficial membrane is often visible, and the anterior end has a wrinkled surface and terminates in a knob, the acrosome (Plate I, fig. 1). These heads appear to be unaffected by ultrasonics or by treatment in the homogeniser, but they are damaged by exposure to a hypertonic saline medium produced, for example, by evaporation of the Tyrode solution in which the spermatozoa are suspended, or by adding some concentrated sodium chloride solution to it. At first a small blister of low refractive index appears at the posterior end of the head. The blister enlarges and bursts and the head uncoils and loses some of its basophil contents (Text-fig. 2). These changes recall the observations by Pollister and Mirsky (14) on the swelling and disintegration of trout spermatozoa heads during treatment with molar sodium chloride solution to extract nucleoproteins.

The proximal centriole cannot be seen in intact heads in the electron microscope, but in heads damaged by a hypertonic medium the centriole appears to be a ring-shaped body surrounding a short conical structure which projects from the middle-piece into the base of the head (Plate I, fig. 5). These structures can with difficulty be distinguished in the light microscope, but were accurately described by Meves (12).

The structure of the middle-piece and tail is not resolved in the light microscope. Broken specimens examined in the electron microscope show

that the axis of both the middle-piece and the tail is composed of a number of fibrils, often seven, each about 500 Å in diameter (Plate I, fig. 4).

The middle-piece is ensheathed by a superficial membrane and by four tape-like fibres spirally wound round the bundle of axial fibrils (Plate I, figs. 2, 3 and 4). Each tape is transversely ridged, the distance from crest to crest being about 400 Å. From observations made with the light microscope (11) it is known that the young spermatid contains four large spherical bodies composed of fused mitochondria, and that these bodies apparently coalesce around the axial fibre to form the middle-piece sheath. It seems probable that the four spirally-wound fibres revealed by the electron microscope have developed directly from the four mitochondrial masses of the young spermatid. The middle-piece sheath ends in the ring-shaped distal centriole (Plate I, fig. 6).

In living eupyrene spermatozoa the tail has a lower refractive index than the middle-piece. In electron micrographs the bundle of axial fibrils in the tail is surrounded by a structureless sheath of low density which

---

In all electron micrographs the specimens have been shadowed with gold-palladium alloy.

PLATE I. Electron micrographs of eupyrene spermatozoa. In each figure, except Fig. 4, the magnification line represents 1 μ.

Fig. 1. Anterior part of head. Fig. 2. Anterior part of middle-piece showing spiral sheath (s) and superficial membrane (m). The head is separated from the middle-piece. Fig. 3. Middle-piece showing ridged spiral sheath. Fig. 4. Broken middle-piece showing axial fibrils and spiral sheath. Fig. 5. Base of head, after exposure to a hypertonic medium, showing proximal centriole (c). Fig. 6. Distal centriole at junction of middle-piece (m–p) and tail (t). Fig. 7. Tip of tail.

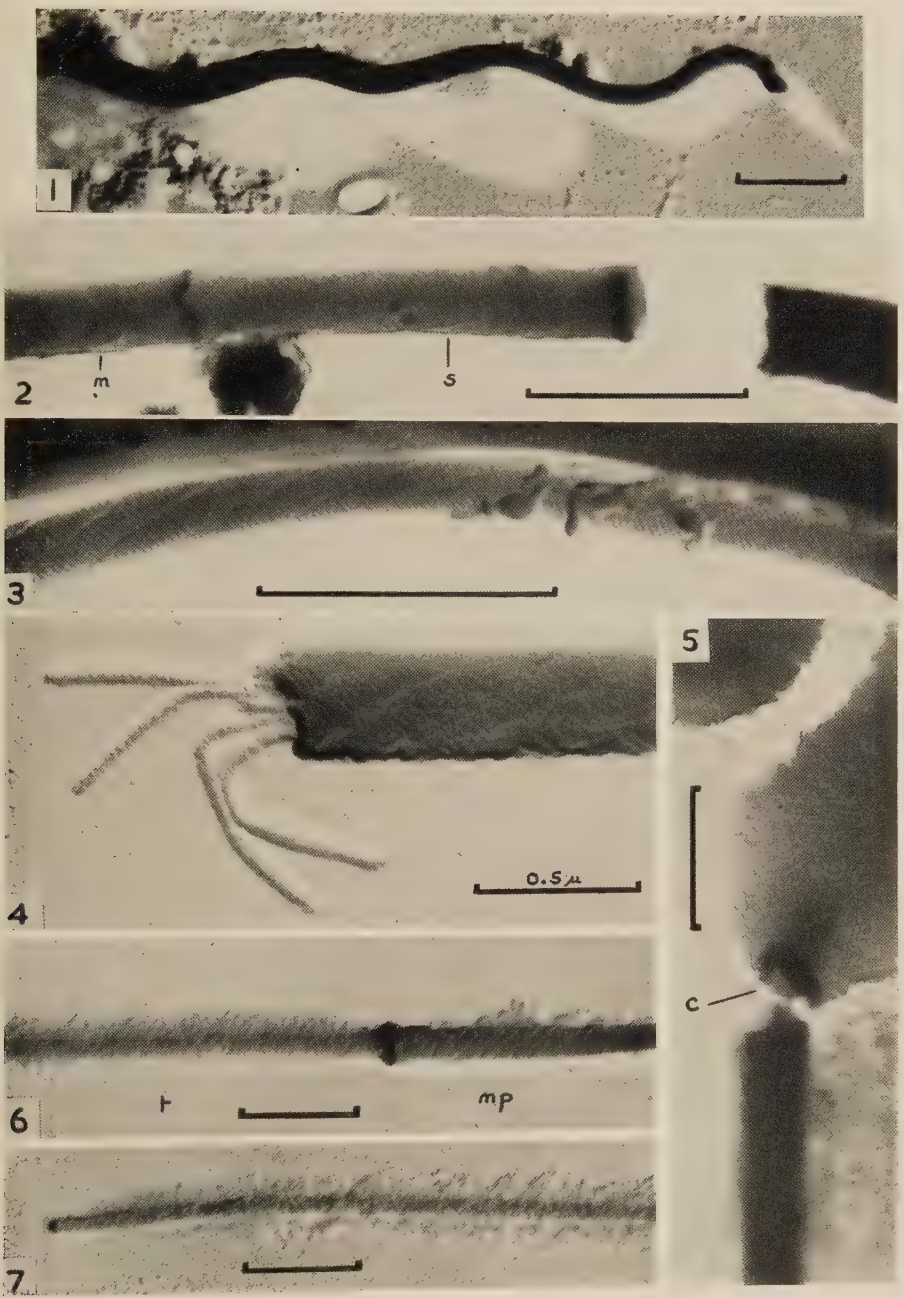
PLATE II. Photomicrographs, taken in phase-contrast microscope, of a suspension of living spermatozoa contaminated with tissues from digestive gland. The granular middle-piece sheaths of the oligopyrene forms are disintegrating to expose the hollow axial cylinders (ac) and the bodies (b) lying inside them. In each figure the magnification line represents 10 μ.

Fig. 1. Three oligopyrene middle-pieces, and head and tail tip of two eupyrene spermatozoa. Fig. 2. Oligopyrene form in which the axial cylinder is splitting into fibres (f). Fig. 3. Oligopyrene middle-piece showing axial bodies (b) and intact granule sheath. Fig. 4. Five oligopyrene middle-pieces and the head of a eupyrene spermatozoon. Fig. 5. Head of eupyrene spermatozoon.

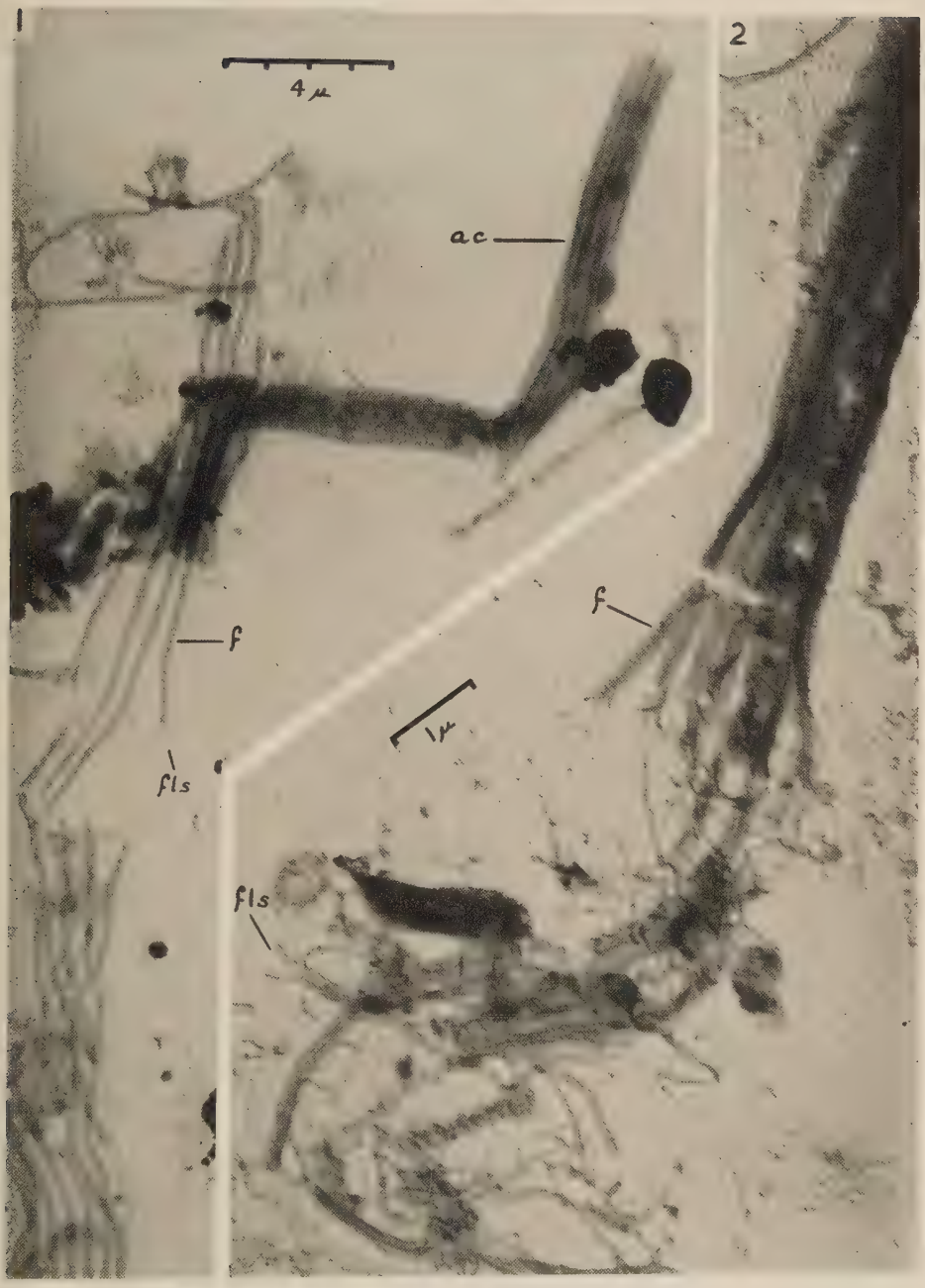
PLATE III. Electron micrographs of middle-pieces of oligopyrene spermatozoa damaged by ultrasonic radiation. The middle-piece sheath has disappeared, the axial cylinder (ac) is releasing its fibres (f), and the fibres have broken and show their constituent fibrils (fls).

PLATE IV. Oligopyrene forms. In Figs. 1–3 the magnification line represents 1 μ.

Fig. 1. Electron micrograph of tails of spermatozoon (sz) (with thick tail sheaths) and spermatid (st) (with thin sheaths). Fig. 2. Electron micrograph of middle-piece of spermatozoon. Fig. 3. Electron micrograph of posterior end of middle-piece of late spermatid, showing structure of granules (g). Fig. 4. Photomicrograph of a transverse section through the middle-pieces of a group of spermatozoa and spermatids. Fixed in Flemming-without-acetic, and stained with iron haematoxylin.


1
2
m
s
3
4
0.5μ
5
c
6
mp
7

1
ac
b
2
f
3
b
4
b
b
ac
5


1
4 μ
ac
f
fls
2
1 μ
f
fls

1
st
sz
2
3
g
4
10μ

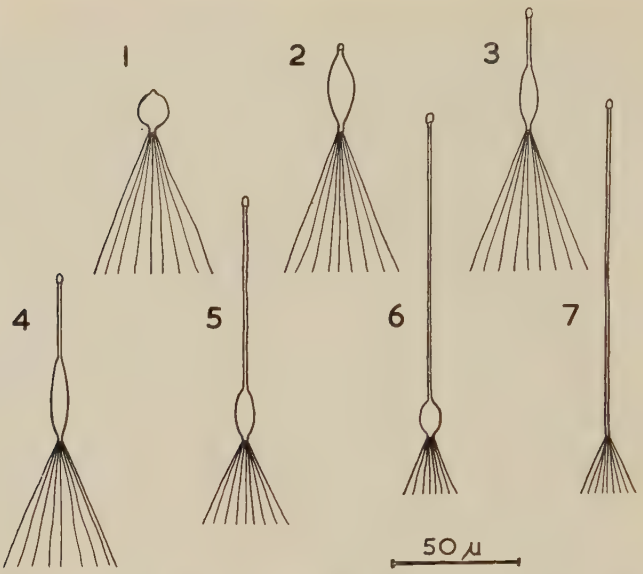
appears to have shrunken and become folded during fixation and drying of the specimens (Plate I, figs. 6 and 7). The sheath narrows at the tail tip where the axial fibre ends in a knob (Plate I, fig. 7). In living spermatozoa one can see that the tail narrows suddenly just in front of the tip, which looks like a short spike. In light microscope preparations stained by the periodic acid—Schiff method (8) for detecting polysaccharides the tail is the only part of the eupyrene spermatozoon which gives a positive reaction. Presumably it is the tail sheath and not the axial fibrils which contains polysaccharide, because the middle-piece is not stained. The reaction is negative after the sections or smears have been incubated in salivary amylase at 37° C for 30 minutes. (The amylase solution was prepared by centrifuging about 20 mls. of saliva and then adding the supernatant fluid to about 50 mls. of phosphate buffer at pH 5.9.)

2. *Oligopyrene Spermatozoa*

Oligopyrene spermatids and spermatozoa from the testis and spermatozoa from fertilised females were examined. The head is shaped like a thimble surrounding the end of the thick middle-piece (Text-fig. 1). The number of tails is commonly about ten but may be as few as 8, or as many as 16. The proximal centrioles lie under the apex of the head, and the distal centrioles at the base of the tail brush.

The tails have the same structure and staining properties as those of eupyrene forms, except that the tips are longer and lack the terminal knob (Plate IV, fig. 1). In young spermatids the tail sheaths are close-fitting and contain no polysaccharide detectable by Hotchkiss's method. Expansion of the sheath and appearance of polysaccharide accompany the decrease in tail length which takes place during development (12). A young spermatid is a bulb-shaped object with a middle-piece, capped by the head, protruding from one pole, and a group of long thin tails emerging from the opposite pole. As the middle-piece elongates the bulb shrinks and the tails thicken and shorten to 40 per cent of their original length (Text-fig. 3). In the electron micrographs there is no obvious change in the thickness of the axial fibre, but the diameter of the sheath increases. This observation suggests that when the tails shorten their axial fibres, but not their sheaths, are drawn forwards into the growing middle-piece.

The middle-piece consists of an axial cylinder surrounded by a sheath which extends from just behind the head-cap to just in front of the distal centrioles. The sheath of living specimens readily disintegrates (see p. 76–77)



Text-fig. 3. Diagrams to show change of shape and shortening of tails during development of an oligopyrene spermatid.

and exposes an axis which appears to be a thin-walled hollow cylinder enclosing a row of spherical or oval-shaped bodies (Plate II). The wall of the cylinder sometimes splits into longitudinal fibres (Plate II, fig. 2). Oligopyrene spermatozoa from the female frequently show naked axial cylinders of similar appearance. Stained preparations of material which has been smeared on a slide and allowed to dry before fixation show naked axial cylinders, the walls of which have split into fibres anchored only by the proximal and distal centrioles. The axial cylinder wall and its constituent fibres are seen in electron micrographs of middle-pieces damaged by ultrasonics or in the homogeniser (Plate III). These fibres are continuous with the axial fibres of the tails. Fragments of membranes in these micrographs suggest that the fibres may be bound together in the cylinder by one or more membranes. Each fibre is composed of about seven fibrils of the same dimensions as the axial fibrils of eupyrene spermatozoa.

The spherical or oval-shaped bodies inside the cylinder are always seen in stripped oligopyrene middle-pieces, but are not visible in undamaged spermatozoa and are not found in sections of spermatozoa in the testis. In sections (Plate IV, fig. 4) a thin strand is seen occupying the centre of a lightly-stained space inside the middle-piece sheath. The wall of the axial

cylinder presumably lines the sheath but is too thin to be detected. We suggest, tentatively, that the bodies inside the axial cylinder of stripped middle-pieces are derived from the axial strand seen in sectioned specimens (see Plate II, fig. 3). Alexenko (1) found that the mitochondria of the young spermatid become incorporated in this axial strand, which he interpreted as the bundle of axial fibres. In the middle-piece of the later spermatid or spermatozoon the axial strand no longer has the staining reactions typical of mitochondria: it is not coloured by Janus green or methylene blue used as vital stains, or by the pyronin in Brachet's methyl green-pyronin method (4); but, like the mitochondrial sheath of the eupyrene middle-piece, it still reduces osmium tetroxide.

The middle-piece sheath is formed from granules which appear for the first time in the spermatocytes. These granules ("granula" of Alexenko (1); "trophochromatic substance" of Artom (3)) are the "mitochondria of Benda" (5), and correspond to the "albuminous bodies" (19, 22) in the atypical spermatozoa of other prosobranchs. They are larger than the mitochondria and differ from them in their staining reactions. The granules in spermatids of all stages of development stain alike. They stain with basic dyes and are Feulgen-negative, and the results of staining by the May-Grünwald and Giemsa method of Jacobson and Webb (9) or Brachet's methyl green-pyronin method, indicate that ribonucleic acids are absent. They are not blackened by osmium tetroxide (even when the specimens are dehydrated in alcohols containing sodium sulphide) and do not stain with Sudan black; therefore, they appear to contain no lipoids. They give a positive reaction to the periodic acid-Schiff test for polysaccharides (8), and the reaction persists after treatment with salivary amylase. They are not stained metachromatically with thionin or with Weigert's resorcin-fuchsin, indicating that the reaction is not attributable to a muco-polysaccharide.

In the bulbous part of the oligopyrene spermatid the granules are scattered through the cytoplasm. In the growing middle-piece they lie in a single closely-packed layer on the surface of the axial cylinder. They become arranged either as a series of rings or in a tight spiral. In electron micrographs each granule appears to consist of smaller granules imbedded in a matrix (Plate IV, fig. 3). In many mature spermatozoa individual granules cannot be distinguished and the middle-piece sheath looks homogeneous in both the light microscope and the electron microscope, but occasional specimens, like that in Plate IV, fig. 2, have surface markings which look like depressions left by granules which have come off the surface of the axial cylinder.

3. *Spermatozoa from the uterus*

Large densely-packed masses of spermatozoa are often found in the uterus near the opening of the oviduct. In these masses the eupyrene spermatozoa are always very active, but the oligopyrene forms usually are non-motile, and the middle-piece and tails are often twisted in a manner never seen in living specimens; the middle-piece sheath is either disintegrating or has disappeared. In some cases, presumably in females dissected soon after copulation, large numbers of oligopyrene spermatozoa are present, and although they show damaged middle-piece sheaths, they are still motile. In other cases no oligopyrene remains can be found amongst the masses of eupyrene spermatozoa. Similar observations were made by Popoff (16), and they indicate that the oligopyrene spermatozoa break down and disappear before ripe ova enter the uterus. We have found that the oligopyrene middle-piece sheath is readily damaged by various means, such as treatment with ultrasonic radiation or in a homogeniser or centrifuge, by hypertonic or hypotonic media, or by exposure to damaged tissues. In the dense clumps of motile spermatozoa in the uterus the oligopyrene forms are likely to be damaged mechanically and may be exposed to a chemical environment which also tends to bring about the break-down of the middle-piece sheath. The significance of the disintegration of this sheath is discussed below.

DISCUSSION

The oligopyrene spermatozoa of *Viviparus* are produced in large numbers by all male individuals throughout the year, and they are found together with the eupyrene spermatozoa in the reproductive tract of the female after copulation. Since they possess only one chromosome they are incapable of fertilising ova in a normal manner, and there is no evidence that they ever stimulate parthenogenetic development or play any role in fertilisation. On the contrary, they probably disintegrate and disappear from the female before fertilisation takes place. This suggests that they have already fulfilled their function, and that one should investigate what part they play during the period between copulation and fertilisation.

These problems have not been investigated in *Viviparus*, but Woodard (22) has made some relevant observations on another freshwater prosobranch, *Goniobasis laqueata*. He has studied *in vitro* the behaviour of ripe spermatozoa from the sperm duct. The eupyrene spermatozoa are non-motile, but the atypical forms (apyrene in this species) are active and soon become entangled

in large dense clumps in which the eupyrene forms are passively incorporated. After a time the eupyrene forms become active and eventually swim away from the clumps, leaving behind them dead apyrene spermatozoa which have lost their middle-piece sheaths. In *Goniobasis* the spermatozoa are transferred to the female in a spermatophore, and Woodard has produced some evidence to show that after the spermatozoa are released from the spermatophore clumping takes place, and then the eupyrene spermatozoa swim away from the clump to the seminal receptacle, where they remain with their heads imbedded in epithelial cells awaiting the arrival of ripe ova.

Woodard's observations suggest that in *Goniobasis* the functions of the apyrene spermatozoa are to prevent premature dispersal of the eupyrene spermatozoa after they escape from the spermatophore in the female reproductive tract, and then to activate the eupyrene spermatozoa and bring about their dispersal. It seems probable that substances released from the disintegrating apyrene middle-piece sheaths are responsible for activating the eupyrene forms, and provide their food supply until they leave the clumps. The reproductive system of male prosobranchs appears to lack accessory glands such as are found in many other animals, and a useful analogy might be drawn between the seminal plasma of a mammal and the atypical spermatozoa of a prosobranch. Reinke (19) foreshadowed this suggestion when he compared the atypical spermatozoa of *Strombus* and other prosobranchs with the atypical ova which nourish the viable ova in the prosobranch ovary. Another observation supports the theory of the nutritive function of atypical spermatozoa. During atypical spermatogenesis some or all of the chromosomes disintegrate in the cytoplasm and, at the same time, there appear large numbers of granules which form the middle-piece sheath of the mature spermatozoa (1, 3). As Reinke (19) has pointed out, fragmentation of the nucleus commonly precedes the formation of yolk or secretory granules in many kinds of cells (see review of Gates (7)).

SUMMARY

1. The structure of spermatids and spermatozoa from the testis and from fertilised females of *Viviparus viviparus* is described. An electron microscope, a phase-contrast microscope, and some recent cytochemical techniques have been used to supplement the older cytological methods.

2. The axis of the flagellum of a eupyrene spermatozoon is a bundle of usually seven fibrils. In the middle-piece four transversely-ridged tape-like fibres are spirally wound round the axial bundle. The thick non-fibrous tail

sheath contains a polysaccharide which is hydrolysed by saliva. The head swells and its basophil contents escape in a hypertonic saline medium.

3. The many tails of an oligopyrene spermatozoon are like the tails of eupyrene forms. During development of an oligopyrene spermatid the tails shorten to 40 per cent of their original length, and at the same time the tail sheath thickens and polysaccharide appears.

4. In the middle-piece of an oligopyrene form the axial fibres from the tail brush are arranged in a hollow cylinder enclosing an axial strand which is perhaps derived from the mitochondria. The middle-piece sheath is composed of granules, distinct from mitochondria, which contain a polysaccharide resistant to salivary amylase, and appear to lack lipoids and nucleic acids.

5. Oligopyrene spermatozoa are transferred to the female at copulation, and whilst they are entangled in large clumps with eupyrene spermatozoa in the uterus the middle-piece sheath disintegrates. It is suggested that the products of the broken-down sheaths may nourish the eupyrene spermatozoa, and that the atypical spermatozoa of prosobranchs are analogous to the seminal plasma of other animals.

REFERENCES

1. Alexenko, B., *Z. Zellforsch.*, **4**, 413 (1926).
2. Ankel, W. E., *Z. Zell.- u. Gewebelehre*, **1**, 85 (1924).
3. Artom, C., *Ric. Morfol.*, **1**, 99 (1920).
4. Darlington, C. D., and La Cour, L. F., The handling of chromosomes. 2nd edition, London, 1947.
5. Gatenby, J. B., *Quart. J. Micr. Sci.*, **63**, 401 (1919).
6. —— The microtomist's vade-mecum. 10th edition, London, 1937.
7. Gates, R. R., *Botan. Rev.*, **8**, 337 (1942).
8. Hotchkiss, R. D., *Arch. Biochem.*, **16**, 131 (1948).
9. Jacobson, W., and Webb, M., *J. Physiol.*, **112**, 2 p. (1951)
10. Kajimoto, N., *Folia Anat. Japon.*, **10**, 441 (1932).
11. Meves, F., *Arch. mikroskop. Anat. Entwicklungsmech.*, **56**, 553 (1900).
12. —— *ibid.*, **61**, 12 (1903).
13. Monné, L., *Bull. Intern. Acad. Sci. Cracovie, Sér. B.*, p. 179 (1930).
14. Pollister, A. W., and Mirsky, A. E., *J. Gen. Physiol.*, **30**, 101 (1946).
15. Pollister, A. W., and Pollister, P. F., *Ann. Acad. Sci.*, **45**, 1 (1943).
16. Popoff, M., *Arch. mikr. Anat.*, **70**, 43 (1907).
17. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
18. Randall, J. T., and Friedlaender, M. H. G., *Exptl. Cell Res.*, **1**, 1 (1950).
19. Reinke, E. E., *Pap. Tortugas Lab.*, **6**, 195 (1914).
20. Selman, G. G., and Wilkins, M. H. F., *J. Sci. Instruments*, **26**, 229 (1949).
21. Tuzet, O., *Arch. Zool. Exptl. Gen.*, **70**, 95 (1930).
22. Woodard, T. M., *J. Exptl. Zool.*, **85**, 103 (1940).

THE CULTURE OF LYMPH NODES *IN VITRO*

O. A. TROWELL

Medical Research Council Radiobiological Research Unit, Harwell, England

Received May 15, 1951

For the purpose of fundamental studies on the sensitivity of lymphocytes to ionizing radiation it was desirable to find some way of keeping a population of lymphocytes alive and healthy *in vitro* for several days. The classical methods of tissue culture in which a fragment of lymphoid tissue is embedded in a plasma clot on the surface of a coverslip or flask are not suitable for this particular purpose because in such cultures some of the lymphocytes die in a few hours and, although some survive for several days, a considerable number of dead or degenerating cells is present at all stages. Attempts to improve this type of technique have not been very successful (35, 38). Suspensions of lymphocytes made by chopping up lymphoid tissue suffer from the same drawback (36).

The purpose of this paper is to describe a method of short-term cultivation of rat lymph nodes *in vitro* which has proved satisfactory and to record some biological properties of lymphocytes which have come to light in the course of the work. Briefly, lymph nodes from young (60 gm) rats are cultured on the surface of cotton wool soaked in the culture medium, the medium is a 2:1 mixture of rat serum and saline with added glucose, and the gas phase is pure oxygen. The respiratory CO_2 coming into the gas phase can be absorbed with NaOH; this is not essential but it permits measurement of the oxygen consumption. Glucose consumption and other metabolic activities are determined by analysis of the medium at the end of the experiment. Small lymph nodes are planted whole, larger ones are first cut in half or into three pieces, the object being to use cultures of about 1 mg wet weight. The living cultures cannot of course be examined under the microscope; at the end of the culture period they are either fixed and sectioned or cut up to make film preparations. There is no outgrowth or outwandering of cells into the culture medium and the method is one of organ culture rather than tissue culture. No claim is made to have achieved either growth or long-term survival of lymph nodes *in vitro*, only that the 'status quo' can be maintained for a few days.

The method owes much to the pioneer work of others in the field of organ culture, particularly that of Strangeways and Fell (37) who introduced the method of culturing whole embryonic organs on the surface of a plasma clot, and that of Parker (31, 32) who cultured small fragments of adult organs in fluid media and was the first to appreciate the high oxygen and glucose requirements of non-embryonic tissues in vitro. The culture of small fragments of adult rabbit lymphoid tissue by the method of Parker, or slight variants of it, has been reported by several workers (12, 29, 33). In my hands, using rat lymph nodes, the Parker method has not given such good results as the one described here and this point will be discussed later.

It is proposed to describe (I) the culture method in detail, (II) the results obtained with it and (III) some experimental results which illustrate the importance of various points in the technique. This last section will include a description of a method of culture on serum-agar which gave satisfactory results, but which, on the whole, is not so convenient as the cotton wool method.

TECHNIQUE

1. *Apparatus.* The lymph nodes are cultured on wet cotton wool in 'culture cups.' These, made from Hysil glass, are 10 mm deep and 12 mm internal diameter. Usually 6–10 cultures are planted in each cup on 0.7 ml of medium. A small groove across the rim of the cup acts as a registration mark with respect to which the cultures are individually located so that each can subsequently be identified (Fig. 1 A).

Each culture cup is housed in a 'culture chamber' which is ultimately filled with oxygen and immersed in a water bath. The chamber is made from three pieces of $\frac{5}{8}$ in. perspex sheet, each $2\frac{1}{4}$ in. square, placed one above the other. The middle piece has a 1 in. diameter hole drilled vertically through its centre after which it is cemented to the base-piece. The top piece forms a removable lid which, in use, is sealed on with lanoline (Fig. 1 B). The lid is held from sliding off by two rubber bands stretched diagonally across from horizontal slots cut across the corners of the base-piece.

A .05 in. diameter hole drilled horizontally through the side wall of the chamber allows the introduction of a hypodermic needle for oxygen filling. A flat ring of filter paper is placed on the floor of the chamber. This is wetted with water or, if it is desired to absorb CO_2, 5 per cent NaOH. The culture cup stands inside the ring and is thus prevented from sliding about on the floor of the chamber. The volume of the gas space, with the cup and medium in position, is 6.5 ml.

In use, the chambers are completely submerged in a water bath maintained at 36° C. The gas hole is left open, for water does not spontaneously leak from the water bath through this long narrow hole into the chamber. During an experiment however, if the respiratory CO_2 is absorbed, water does enter the chamber and the volume entering is equal to that of the oxygen consumed by the cultures. The total oxygen consumption can be measured in this way as will be described later.

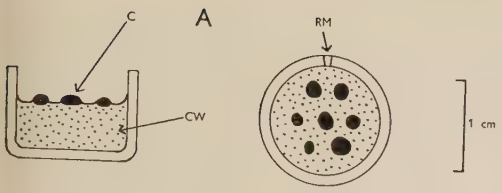


Fig. 1 A.

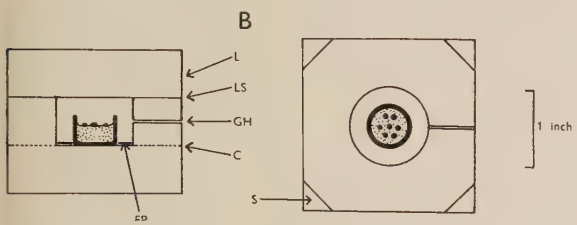


Fig. 1 B.

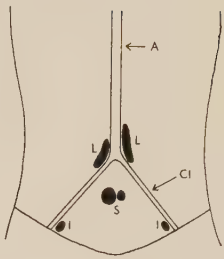


Fig. 2.

Fig. 1. A. Culture cup, vertical section and plan. C = culture, CW = cotton wool soaked in medium, RM = registration mark.

Fig. 1. B. Culture chamber, vertical section and plan. C = cemented joint, FP = filter paper ring, GH = gas hole, L = lid, LS = lanoline seal, S = slots for rubber bands.

Fig. 2. Position, shape and size of lumbar (L), sacral (S) and iliac (I) lymph nodes on the posterior abdominal wall of a young rat. A = aorta and vena cava, CI = common iliac vessels.

2. *Materials. Rats.* Except where otherwise stated a pure Wistar strain bred in this laboratory has been used. The rats were housed at 70° F and fed on commercial rat nuts supplemented with brown bread, marmite and cod liver oil. They were not starved before use.

Cotton Wool. The surface of the cotton wool in the culture cups must be perfectly level and even and the wool of uniform texture throughout, otherwise lakes and hills will appear when it is wetted with medium. This cannot be achieved by packing loose wool into the cups. It is also necessary to purify the wool by removing the fat and any water-soluble constituents. The following method of preparation has proved satisfactory. The starting material is 'absorbent cotton wool rolls,' 1 ½ in. long and ½ in. diameter (Johnson and Johnson Ltd.). Each roll is sewn up tightly in a double layer of surgical gauze to prevent it from unravelling in the cleaning process. The rolls are soaked in ether for 24 hours, after which they are handled only with forceps or rubber gloves. The ether is dried off and they are then boiled in several changes of glass distilled water for 3 hours, and dried in the oven. Each roll, with the gauze still on, is then carefully cut into slices 5 mm thick with a safety razor blade. A slice is placed in each culture cup and these are then dry sterilised at 120° C.

Serum. This is obtained from rats weighing 150–200 gm, 2–3 months old. Under ether anaesthesia the chest is widely opened with full asepsis and blood is taken from the right ventricle with a needle and syringe. The maximum amount of blood is withdrawn and the animal, of course, does not recover. The blood is centrifuged at once. After coagulation, the plasma clot is broken up with a glass rod, the tube recentrifuged and the serum at once drawn off.

Glucose saline. This is 0.7 gm NaCl and 0.6 gm glucose dissolved in 100 ml of glass distilled water.

Medium. The medium used both for preliminary washing and final culturing is 2 vols. of serum plus 1 vol. of glucose saline. The final glucose concentration is 0.2 per cent higher than that of the original serum and is usually around 0.4 per cent.

Cleaning and sterilising. All glassware is cleaned with soap, rinsed in hot water, stored in pure nitric acid for several days, then well washed and dried in the oven at 120° C. Gauze and wool, used for plugging tubes and pipettes, is cleaned by soaking in ether then boiling in distilled water. Glassware and instruments are sterilised at 120° C dry for 1 hr., glucose saline at 100° C for ½ hr. The perspex chambers are cleaned with petrol ether to remove lanoline, then washed in cold water and dried. They are sterilised by immersion in ether for a minute or so, the ether is then dried off and the chambers are stored in a sterile tin.

3. *Procedure.* The lymph nodes used are the lumbar, the sacral, and sometimes the iliac. In order to secure lymph nodes of the right size, attention is paid to the weight of the rat rather than its age. Rats between 50 and 90 gm can be used, but 60 gm is the optimal size and in our colony this weight is reached at about the age of 4 weeks. These particular nodes are chosen because they are of convenient size, they are very constant in position (except that one sacral node is sometimes absent) and they can be removed aseptically without any risk of contamination from the skin. Furthermore these nodes at this age consist of quite uniform lymphoid tissue, there is as yet neither differentiation into cortex and medulla nor development of follicles. In older animals a variable number of follicles is present which means more initial variation among the cultures, while in younger animals quite large areas of the node may be devoid of lymphocytes. The lumbar nodes sometimes contained superficial patches of fresh (red) or old (brown) blood. 'Occasional red mottling' of the lumbar nodes in normal rats has also been noted by Macmillan (24), though she, rightly, excluded them from the category of haemolymph nodes. The appearance of blood patches in the lumbar nodes following the injection of carcinogenic hydrocarbons has been reported by Laznitski and Woodhouse (23), but they found no such patches in normal nodes.

It has been found convenient to use 6 rats, all of one litter, for each experiment. They are killed one at a time as required. Each rat yields 6–10 cultures and all the cultures from the first rat are planted on one cup and the setting up of that chamber is completed before the next rat is embarked upon. So there are finally 6 chambers and a total of 40–60 cultures, each chamber corresponds to an individual rat and each culture is identifiable by reference to the registration mark on the cup. Such an experiment requires 5 ml of serum and usually 2 donor rats.

The procedure for setting up each chamber is as follows. The rat is decapitated and pinned out on its back on a sheet of cork mounted on a lead base. The rat and cork are liberally swabbed with alcohol. The skin is widely reflected from the trunk. The abdomen is then opened with sterile instruments and the intestines carefully drawn aside right out of the abdomen. The lymph nodes are now readily visible (Fig. 2) though it is advantageous to retract the rectum with a bull-dog clip and in females to divide and reflect the right uterine horn. Before attempting to remove the nodes it is important to cover them with a few drops of medium so that, in effect, they are dissected out under a layer of fluid. If a node is dissected dry it may lose, by

evaporation, 20 per cent of its weight in two minutes and this leads to bad cultures. The nodes are not handled at all with forceps. They are freed by picking up and incising the adjacent connective tissue and are transferred with adhering tags of tissue to a pool of medium in a large cavity slide. There they are cleaned free of connective tissue and fat and the larger ones are cut in half with cataract knives. The sacral and iliac nodes are spherical and are usually used whole, but sometimes cut in half. The lumbar nodes are sausage shaped and are usually cut in half or into three.

The culture cup is now prepared by pipetting on to the cotton wool enough medium to moisten it without leaving any free fluid on the surface. If the surface of the wet wool appears at all uneven this can now be rectified by gently kneading it all over with the tip of the pipette. A little more medium is added so that there is a thin film of free fluid over the surface of the wool. The cultures are now planted, after which the amount of medium on the wool is carefully adjusted, so that while there is an easily visible glistening film of fluid over the whole surface, no gravity flow of fluid occurs when the cup is tilted and the cultures stick well up into the air.

Having put a filter paper ring into the chamber and wet it with 0.2 ml of water or 5 per cent NaOH, the cup is inserted, the lid sealed on with lanoline and the rubber bands applied. The chamber is at once placed in the 36° C water bath. After 15 mins., by which time the temperature inside the chamber has reached 32° C, 60 ml of oxygen is passed through the chamber. The oxygen first passes through a long glass tube tightly packed with wet glass wool and immersed in the water bath; this ensures that the gas is saturated with water vapour at 36° C. The flow is set at 60 ml per min. and the oxygen is delivered by a hypodermic needle inserted through the gas hole into the central space of the chamber. The displaced air escapes along the side of the needle (which is much narrower than the hole) and bubbles up through the bath water. It has been found by direct analysis of the gas in the chamber that the passage of 20 ml of oxygen under the conditions just described suffices to raise the oxygen percentage to 97 or 98, so 60 ml allows a big margin of safety.

At the end of the culture period (usually 48 hr.) the cup is removed and after wiping dry on the outside the whole cup and contents is weighed on a torsion balance. The cultures are then lifted off and quickly transferred to the celluloid pan of a 10 mgm torsion balance, any excess of fluid is mopped up with filter paper and the total weight of the cultures determined. The cultures are then put in serum for the time being. A small drop of medium is sucked off the wool and its pH determined by the B.D.H. Capillator. Further samples are taken for glucose or other estimations, after which the wool is washed, dried and weighed. Knowing the tare weight of the cup, the weight of fluid medium can now be found by difference. The volume of water in the chamber itself is also measured.

4. *Histological Examination of Cultures.* The main interest was to find out how many dead lymphocytes were present in the cultures at the end of the experiment. In normal lymph nodes (*in vivo*) about 1 per cent of the lymphocytes are dead, and successful cultures can be expected to approach this figure. When lymphocytes die their nuclei undergo pyknotic degeneration and the pyknotic (dead) cells can easily be recognised and counted. In a few cases the cultures were fixed in Susa and serially sectioned in paraffin, but this is too time-consuming when large numbers of cultures are handled. Wet-fixed film preparations have been routinely used throughout this work and have proved very satisfactory.

The culture is placed in a small drop (0.002 ml) of serum on a slide and cut up into 4–6 fragments with cataract knives. After being well stirred, the fragments are removed on the point of the knife, the remaining suspension of free cells is quickly spread with the knife to make a thickish film about 2 cm in diameter, and the slide at once plunged into Susa fixative. The wet film is instantaneously fixed and is not washed off by the fixative. This method of wet-fixation of films in Susa was introduced by White (43) who used it for the study of bone-marrow cells and pointed out its advantages for the preservation of nuclear structure and of nucleoproteins in general. It may be mentioned that air-dried films were found unsatisfactory because some of the lymphocytes disrupted during drying, the cells flattened in varying degree, nuclear detail of lymphocytes was entirely lost and the recognition of cell types and the early stages of nuclear degeneration was much more difficult than in wet-fixed films. After 30 min. fixation and 30 min. in running water, the films are stained in Mayer's Haemalum (the later formula) (27) for 2 min. They are then washed in running tap water 15 min., dehydrated in 'cellosolve' (ethylene glycol monoethyl ether) and mounted in cellosolve-balsam. By using cellosolve, the cell shrinkage which occurs in xylol and other clearing agents is avoided.

All available staining methods were tried and Haemalum was chosen because the staining time is not critical ($\frac{1}{2}$–5 min.), there is no risk of overstaining and all the cells are evenly stained despite the varying thickness of different parts of the film. Further, it does not stain the serum background and it differentiates ribonucleic acid (nucleolus and basophil cytoplasm) which stains pale mauve, from deoxyribonucleic acid (chromatin) which stains dark blue. Cytoplasmic basophilia is easily appreciated and assessed (e.g. Fig. 12). The stain was made up from solid haematoxylin obtained from G. T. Gurr; haematoxylin from some other sources was found unsatisfactory. Staining methods which involve differentiation, such as Romanowsky, Methyl Green-Pyronin, are unsatisfactory because in these rather thick films the differentiation always occurs unevenly.

It is widely believed that Zenker-formol is an excellent and, in fact, the best fixative for lymphocytes, but in my experience it has not proved to be so. Comparison of the lymphocytes in wet-fixed haematoxylin-stained films with living lymphocytes examined under phase contrast showed that the nuclear shape and structure in particular is much better preserved by Susa than by Zenker-formol. The 'chromatin pattern' of the nuclei of living lymphocytes examined under phase contrast resembled very closely that seen in the same cells after Susa fixation and haemalum staining.

The great majority of the cells in the films are small lymphocytes, but there are also present medium lymphocytes and large lymphocytes (in the terminology of Maximow), pyknotic lymphocytes, reticular cells (including macrophages) and

---

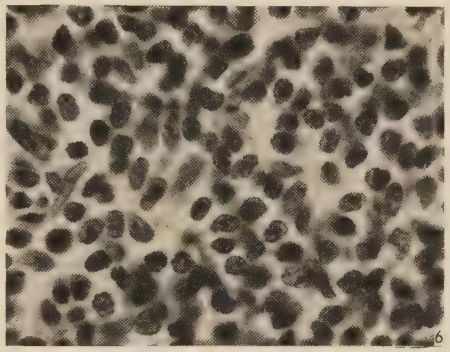
Figs. 3–6. Paraffin sections of lymph nodes. Fixed in Susa and stained with Haemalum. Fig. 3—a normal node, Fig. 4—a two-day culture, Fig. 5—a four-day culture, all × 44. Fig. 6—the four-day culture. × 740.

Figs. 7–14. Typical cells from two-day cultures. Films wet-fixed in Susa and stained with Haemalum. × 1050.
Fig. 7. Small Lymphocytes. Fig. 8. Medium Lymphocytes. Fig. 9. Large Lymphocytes. Fig. 10. Reticular cells (macrophages). Fig. 11. Cells intermediate between reticular cells and large lymphocytes. Fig. 12. Plasma cells. Fig. 13. Mitoses (large lymphocyte and small lymphocyte). Fig. 14. Pyknotic lymphocytes.

3

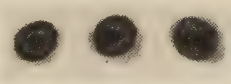
4

5

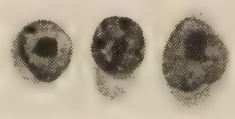


6

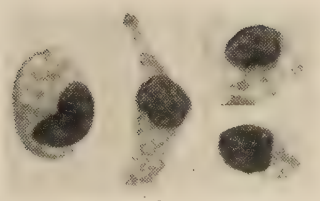
7



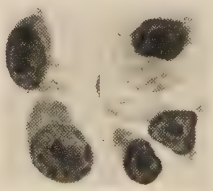
8



9



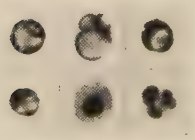
10



11

12

13



14

occasional monocytes, plasma cells and polymorphs (Figs. 7–14). A differential count is made on each film, counting and classifying every cell in a succession of oil immersion fields until a total of 500 lymphocytes is reached. The most important information obtained is the number of pyknotic (dead) lymphocytes present expressed as a percentage of the total lymphocytes; this will be called the 'pyknotic count' (P. C.), and in normal cultures it is about 2 per cent. In a series of 1,700 cultures the P. C. has proved a reliable and accurate method of assessing the health of cultures and also of measuring quantitatively the lethal action of chemical substances and ionizing radiations on lymphocytes in vitro.

RESULTS

A. *Histology of cultures*

It is first necessary to mention some histological features of the normal nodes from which the cultures are derived. These nodes (lumbar and sacral of 4 week-old rats) contain no follicles (germinal centres) and very few mitotic cells. They consist simply of a network of reticular cells closely packed with lymphocytes (Fig. 3). Differential counts of wet-fixed films from 58 normal nodes are shown in Table I (A). It is evident from the low mitotic rate and the paucity of immature cells (medium and large lymphocytes) that very little lymphopoiesis is going on in these nodes. Other workers have reported slightly higher figures for lymph nodes in rats of this age (1, 19) but, even so, they considered that the rate of lymphocyte production was barely sufficient to account for the normal growth in size of the nodes and it is generally agreed that at this age the thymus is the main source of the blood lymphocytes.

Turning now to the cultures, Fig. 4 shows a section of a 2-day culture and Fig. 5 a 4-day culture, with Fig. 3, a normal node, for comparison. There is no great difference in appearance and in particular it should be noted that there has been no appreciable reduction in the total number of lymphocytes as a result of culture, because this is a point which cannot be proved from the films. Fig. 6 shows part of the 4-day culture under higher magnification and exhibits healthy lymphoid tissue indistinguishable from that of a normal lymph node.

282 normal 48 hr. cultures have been examined by the film technique and the results are shown in Table I. Examples of the different cell types found in the films are shown in Figs. 7–14. Each experiment represents the nodes from one litter of rats cultured in one batch of serum, the litter and the batch of serum being different in each experiment.

In 4 of the experiments the chambers were filled with 3 per cent CO_2 in oxy-

TABLE I

Mean Differential Cell Counts of Normal Lymph Nodes and 48 hr. Cultures.

A. Normal lumbar and sacral nodes of 60 gm. rats.
B. 48 hr. cultures on cotton wool - serum - glucose saline. Each entry refers to a single experiment using several chambers, litter-mate animals and the same batch of medium.

The results are given as per cent of total lymphocytes. The per cent figures for each culture or node were separately computed from a total of 500 lymphocytes counted in each case. The figures given are the mean values for each experiment, obtained by averaging the figures from the number of cultures stated.

	Gas phase	No. of nodes of cultures	Medium Lympho-cytes	Large Lympho-cytes	Pyknotic Lymphocytes		Lympho-cyte Mitoses
					Mean	S.E. of mean	
A. Normal Nodes		58	0.69	0.08	1.04	0.07	0.014
	O_2	26	0.11	0	1.65	0.26	
[1]	»	15	0.08	0	2.28	0.23	
	»	23	0.10	0.02	1.65	0.30	
	O_2, CO_2 absorbed	25	0.25	0	2.11	0.36	
	»	31	0.22	0	1.95	0.26	
B. Cultures	»	29	0.32	0.01	1.02	0.09	
	»	18	0.13	0.02	0.67	0.08	
	3 p.c. CO_2 in O_2	22	0.49	0.05	1.39	0.22	
[2]	»	18	0.30	0.03	1.10	0.17	
	»	47	0.35	0.06	1.39	0.10	
	»	28	0.37	0.03	2.45	0.45	
Average of all Cultures		282	0.26	0.02	1.61	0.08	0.005

gen, in the other 7 pure oxygen was used and in 4 of these the respiratory CO_2 was absorbed with NaOH. It will be seen that despite considerable differences in the pH of the medium the results were substantially the same; they have therefore all been regarded as normal 48 hr. cultures and the average P.C. for the series is 1.61 (S.E. 0.08). This is to be compared with the average P.C. of 1.04 (S.E. 0.07) for corresponding lymph nodes *in vivo*. There are, therefore, a few more dead cells in the cultures than in normal nodes, but the results are fairly consistent and it is considered that the culture method reasonably fulfills the purpose for which it was designed.

Table I also shows that the proportion of medium and large lymphocytes is less than in the normal nodes. It might be that these immature cells survive less well *in vitro* than the mature small lymphocytes, but as pyknotic medium

[1] Tyrode solution used instead of glucose saline.
[2] Serum from Compton rat.

TABLE II

Pyknotic Counts of 4, 6 and 8 day cultures on cotton wool - serum - glucose saline. Gas phase O_2, CO_2 absorbed. Data presented as in Table I.

Brackets enclose parallel experiments using litter-mate animals and the same batch of serum.

Age of cultures (days)	Medium changed	No. of cultures per cup	Total No. of cultures	Pyknotic count		Final pH of medium
				Mean	S.E. of mean	
4	—	2	8	1.62	0.39	—
4	—	6	18	1.62	0.32	7.2–7.3
4	—	6	12	1.48	0.11	7.2–7.3
4	transplanted at 2 days	8	24	2.72	0.56	7.6–7.8
4	medium renewed at 2 days	6	6	3.53	1.06	7.4
6	—	4	16	17.53		6.9–7.0
8	transplanted at 4 days	4–5	24	60.12		7.1–7.3
8	transplanted at 4 days	5–6	11	61.03		—

or large lymphocytes were rarely, if ever, seen, a more likely explanation is that, *in vitro*, the maturation of these cells to small lymphocytes goes on normally but there is no formation of new immature cells to take their place. The very low mitotic count in the cultures (1 in 20,000) would support this interpretation.

The number of reticular cells varied greatly from film to film; monocytes, plasma cells and polymorphs occurred in some litters but not in others; so the average figures for these cells are not very significant and are omitted.

Analysis of the figures for the individual cultures showed that none of the following factors appreciably affected the results—the size of the culture (range 0.2–1.5 mg), the origin of the culture (lumbar, sacral or iliac), whether half node or whole node, the number of cultures per cup (range 4–14). The variation between individual cultures may be judged from the results of two typical experiments set out in Fig. 17. This variation will be discussed in a later section.

In a few experiments cultures were maintained for 4, 6 and 8 days to see how long they would survive. The results, given in Table II, show that at 4 days the cultures were still as healthy as they normally are at 2 days. The best results were obtained when the cultures were left undisturbed; when the medium was changed after 2 days, either by sucking off the old medium and replacing it with fresh, or by transplanting the cultures to a new cup of fresh medium, the results were not quite so good though still fairly satis-

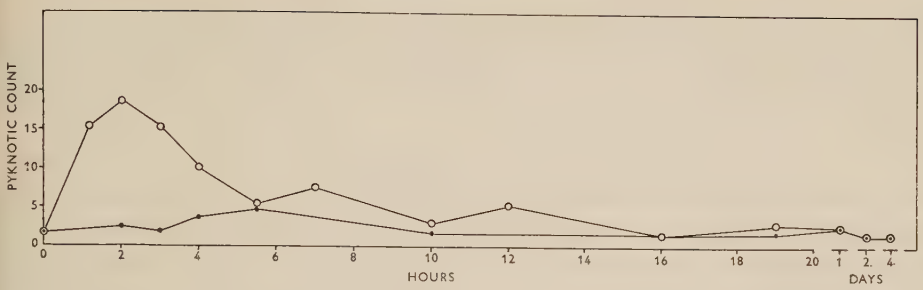


Fig. 15. Pyknotic counts of cultures after various periods of time *in vitro*. Each point is the average of 6 or more cultures.

• = whole nodes, ○ = half nodes, ⊙ = whole and half nodes not separated.

factory. Cultures left undisturbed for 6 days gave varying results. Some of the cultures were quite healthy but in others there was a good deal of lymphocyte pyknosis so that the average results were not satisfactory. Cultures transplanted on the 4th day and maintained for 8 days were all unsatisfactory. It is concluded that the present technique can be relied upon to maintain all the cultures in a healthy state for 4 days but no longer.

In another series of experiments cultures were sacrified and examined after varying periods *in vitro* ranging from 1 to 24 hr. and the results are shown in Fig. 15 together with the results of the 2-day and 4-day cultures. The zero point was obtained from cultures put up in the routine manner and then sacrificed at once without incubation. Half-node cultures and whole-node cultures are plotted separately and it is seen that in the half nodes there is an outbreak of lymphocyte degeneration during the first hour or two which does not occur in the whole nodes. This is interpreted to mean that in the half nodes a number of the lymphocytes are dead or damaged (but still normal in appearance) at the start of the experiment, and that these undergo the normal process of pyknotic degeneration during the first hour or two of culture and then slowly disappear, disappearing completely within about 16 hr.

It seemed that the death of these lymphocytes must be associated in some way with the trauma inflicted when the node was divided by the knife and this was confirmed in sections of 2 hr. cultures which showed a zone of pyknotic lymphocytes extending a little way in from the cut surface. In 48 hr. cultures these pyknotic cells have disappeared and the cut surface is very difficult to recognize because a new capsule has formed. The trauma must involve something more subtle than simple mechanical disruption of the cells because in sections of half nodes fixed 15 min. after transection,

the cells along the cut surface are still quite normal in appearance. The use of half nodes has not, on this account, been discontinued, for after 16 hrs. the early degenerating cells have disappeared and the cultures are indistinguishable from those of whole nodes.

The disappearance of these pyknotic lymphocytes might be ascribed to dissolution or to macrophage activity. However, in the case of pyknotic degeneration induced in cultures by other means (e.g. anoxia, X-radiation), in which case the pyknotic cells are scattered throughout the culture, it has been observed that the pyknotic cells may remain, scarcely diminished in number, for 24 hr. and often longer. It is therefore thought more likely that in the half nodes the pyknotic zone is as it were sloughed off by the development of a new capsule in the healthy tissue underneath.

It is easy to recognize successive stages in the process of pyknotic degeneration and in 48 hr. cultures all stages are represented from very early pyknoses to very old ones. This indicates that during culture there is a continuous, but very low mortality of lymphocytes and that the old pyknoses do eventually disappear, thus maintaining a steady balance of about 2 per cent of pyknotic cells present. In the virtual absence of mitosis there must therefore occur a steady reduction in the total number of lymphocytes present, but this is so slight as to be inappreciable in 48 hr. After 4 days however, there is some obvious decrease in the size of the cultures.

It is of some interest to know what time usually elapses between the death (or injury) of a cell and the first appearance of histological degeneration. Very little information exists on this point because it is not easy, experimentally, to kill cells at a precise moment without at the same time coagulating and fixing them, in which case of course the process of degeneration does not occur. So it is worth pointing out that in the experiments just described the cells all degenerated within 2 hr. of the infliction of the trauma.

In view of the fact that under certain conditions in hanging-drop cultures, small lymphocytes have been observed to transform into macrophages (3, 7, 26, 30) it is worth recording that no sign of such transformation was observed in the cultures described here. Cells intermediate between small lymphocytes and plasma cells were occasionally seen and an apparent transformation of reticular cells into large lymphocytes has been observed in a few cultures. In these cultures cells intermediate in appearance and covering all stages between reticular cells and large lymphocytes were found (Fig. 11). Such cells were not seen in normal nodes. The only evidence that the transformation was in the direction reticular cell to large lymphocyte, and not vice versa, was that such cultures contained more medium and large

lymphocytes than usual. A similar development of large lymphocytes from reticular cells was clearly demonstrated by Maximow (25) in hanging-drop cultures of rabbit lymph node.

B. *Metabolism of cultures*

Oxygen and glucose consumptions were measured in some of the experiments described in the previous section. The methods employed have already been indicated and it remains to add some further details. When oxygen consumption was measured a blank chamber was always set up and run as a control. This chamber was set up exactly like the others except that no cultures were put on the medium. At the end of the experiment the small volume of water which had entered the blank chamber was deducted from the volume which had entered each culture chamber in order to obtain the true oxygen consumption of the cultures. This control chamber corrected for changes in temperature and barometric pressure occuring during the experiment and also for any uptake of oxygen by the medium or the walls of the chamber. Glucose was determined by the method of King and Garner (21), a photo-electric absorptiometer was used, all determinations were done in duplicate and the duplicates generally agreed within 5 per cent. The total weight of the cultures and the volume of medium in each cup was determined at the end of the experiment and the oxygen and glucose consumptions per mg of tissue (wet weight) per hr. were calculated.

The results are summarised in Table III. No high accuracy can be claimed for these results for they are subject to some known sources of error which it will be well to mention. In the first place there always is some disappearance of oxygen in the control chamber. This is partly due to consumption of oxygen by the medium (? oxidation of lipoid), partly to oxidation of the lanoline and partly to diffusion of oxygen into the perspex. Although in theory these losses are corrected for by subtraction of the blank, they will in fact vary somewhat from chamber to chamber. Another difficulty concerns the glucose. In most of the experiments CO_2 was absorbed so that the medium was initially quite alkaline (pH 8.6) and the stability of glucose at this pH may be questioned. It was in fact always found that the glucose concentration in the blank chamber (initial pH 8.6, final pH 8.8) had apparently risen somewhat at the end of the experiment, presumably due to the breakdown of glucose to more actively reducing substances such as hydroxymethylglyoxal as a result of the alkalinity. In the culture chambers, although the pH is initially 8.6 it rapidly falls during the experiment (owing to lactic acid production) so

TABLE III

Total Oxygen and Glucose Consumptions of 48 hr. cultures.

Each entry corresponds to a single chamber containing cultures derived from one animal. Each 'experiment' comprises several parallel chambers (bracketed) using litter-mate animals and the same batch of serum.

In the last two experiments the concentration of glucose in the glucose saline was less than the usual 0.6 per cent.

Culture period (hr.)	Total weight of cultures (mg)	O_2 consumed (µl) (N.T.P.)	µl O_2/mg/hr.	Initial glucose conc. (mg/100 ml)	Final glucose conc. (mg/100 ml)	Glucose consumed (mg)	Glucose oxidized (theoretical) (mg)	µg glucose/mg/hr.	Initial pH	Final pH	
47	3.7	264	1.53	380	268	0.68	0.35	3.94	8.6	8.0	
47	4.8	448	1.98	380	292	0.62	0.60	2.75	8.6	8.2	
48	3.4	231	1.42	380	300	0.6	0.31	3.70	8.6	8.2	
48	4.6	318	1.44	343	211	0.85	0.43	3.85	8.6	8.0	
50	5.5	386	1.40	343	198	1.02	0.52	3.64	8.6	7.8	
46	7.7	461	1.31	397	191	1.62	0.62	4.60	8.6	7.8	
46	6.5	466	1.57	397	226	1.46	0.63	4.90	8.6	7.8	
47	5.1	322	1.34	397	187	1.16	0.43	4.84	8.6	7.5	
47	5.5	470	1.84	397	229	1.00	0.63	3.91	8.6	7.6	
49	4.0	243	1.25	397	277	0.76	0.33	3.90	8.6	7.6	CO_2
46	5.24	303	1.25	393	314	0.59	0.41	2.45	8.6	8.4	absorb
47	3.93	191	1.04	393	323	0.59	0.26	3.20	8.6	8.2	
47	9.0	573	1.36	372	306	0.51	0.77	1.21	8.6	8.1	
47	11.2	688	1.31	372	228	1.07	0.92	2.04	8.6	7.4	
47	12.0	382	0.68 [1]	372	350	0.13	0.51	0.23 [1]	8.6	7.6	
47	7.9	451	1.22	372	221	1.02	0.6	1.81	8.6	8.4	
49	12.6	512	0.83	372	231	1.08	0.69	1.83	8.6	7.5	
48	8.3	660	1.66						8.6	7.6	
48	8.2	670	1.70						8.6	7.8	
48	8.0	480	1.25						8.6	8.2	
48	3.1			238	196	0.3		2.02	7.8	7.0	
48	9.6			238	128	0.78		1.70	7.8	7.2	
48	11.0			238	100	0.97		1.84	7.8	7.1	
48	3.2			150	102	0.34		2.21	7.8	7.0	CO_2 n
48	11.2			150	50	0.70		1.30	7.8	7.2	absorb
48	12.6			123	48	0.52		0.86	7.8	7.4	
48	12.0			250	124	0.88		1.53	7.8	7.0	
48	15.5			250	90	1.12		1.51	7.8	7.1	
Average			1.37					2.63			

[1] No explanation can be offered for the low results in this chamber, histologically the cultures appeared normal (P.C. = 1.22).

this error cannot be allowed for simply by subtracting the result of the blank, and there is no simple way of computing it.

The average of the 20 oxygen consumptions was 1.37 μl/mg wet wt./hr. The average wet weight/dry weight ratio for these lymph nodes was found to be 4.0, and multiplying by this factor gives a QO_2 of 5.48. The QO_2 of lymph-node slices over a period of 1–2 hr. survival has been measured by others, using conventional manometric methods. There appear to be no data for the rat, but the following values have been reported for young mice—4.6 (40), 5.34 (41) and 6.28 (16). For human lymph nodes, values of 5.7 (18) and 3.8 (42) are reported. In the rabbit rather higher figures have been found —8.4 for lymph nodes and 8.04 for the appendix (34). The oxygen consumption reported here for lymph-node cultures over a 48 hr. period is therefore in general agreement with the published figures for lymph-node slices over a 2 hr. period.

The glucose consumption (in contrast to the oxygen consumption) varied considerably from one experiment to another, but in any one experiment the values from the individual chambers were fairly consistent. This experiment to experiment variation is likely to be due to the serum used for the culture medium. Different samples of serum may well vary in their content of insulin, corticosterone and anterior pituitary hormones, all of which are known to influence the rate of glucose metabolism *in vitro* (6). There was no obvious correlation between glucose consumption and glucose concentration over the range 90–397 mg/100 ml, but in the two cases in which the glucose concentration fell to 50 and 48 mg/100 ml respectively, the consumption was lower than that in parallel chambers.

From the oxygen consumption it is possible to calculate the maximum amount of glucose which could have been oxidized to CO_2 and water (1 ml O_2 = 1.34 mg glucose). The actual amount so oxidized may be less than this because the oxygen consumed may not all be used for the oxidation of glucose, though the R.Q. of 0.93 found by Redfield and Barron (34) suggests that nearly all of it is. The results of this calculation are listed in Table III and it is apparent that in nearly all cases more glucose was consumed than can be accounted for by oxidation. The balance is presumably due to breakdown of glucose to lactic acid and represents a considerable aerobic glycolysis. This relatively high aerobic glycolysis of lymphoid tissue has also been noted by workers using the tissue-slice technique (18, 34).

Table III shows that the pH of the medium always falls quite considerably during the period of culture, even though all the CO_2 is absorbed, and it should be added that in the blank chambers the pH rises from 8.6 to 8.8

during the 48 hr. period. Under such conditions (absorption of CO_2) a fall in pH can only be due to the production of fixed acids by the cultures. In view of the aerobic glycolysis already demonstrated it is probable that the pH change is mainly due to lactic acid. Rough analyses for total protein and total lipid showed that no striking change in the concentration of either of these components of the medium occurred during the period of culture.

These observations are sufficient to show that the cultures maintain a fairly normal carbohydrate metabolism *in vitro*. More accurate methods for the measurement of these and other aspects of the culture metabolism are being developed.

EFFECTS OF VARYING THE CONDITIONS OF CULTURE

Lymph nodes have also been cultured under conditions differing from those so far described—for example the composition of the medium and the gas phase has been varied—in an attempt to discover the optimal conditions for lymphocyte survival *in vitro*. In most, but not all cases, the results, as assessed by the P. C., have been inferior to those obtained with the established technique. The results are however of interest because they demonstrate quantitatively the relative importance of most of the factors involved in the established technique, and they constitute in fact the experimental data from which the technique was gradually evolved.

1. *Oxygen.* Some cells, notably fibroblasts and macrophages, can survive *in vitro* under highly anoxic conditions, but this is not the case with lymph-node lymphocytes. The problem of oxygen supply can be approached theoretically. As already shown, lymph nodes *in vitro* consume rather more than their own volume of oxygen per hour and this would correspond to removal of all the dissolved oxygen from forty-four times their own volume of medium (which almost equals the volume actually used) in one hour. It is clear therefore that the oxygen supply must be maintained by diffusion from the gas phase and that the rate of diffusion of oxygen through the medium and through the tissue will be a factor which limits the size of culture that can be maintained.

Consider the culture to be a sphere of radius r cm and let the concentration of oxygen at the surface of the culture (measured in atmospheres pressure) be C. As oxygen diffuses into the culture some of it is consumed by the cells and the oxygen concentration at the centre will be lower than at any other point. Consider now the limiting case in which the oxygen concentration at the centre just reaches zero; the limiting radius for such a case is given by the following formula (14)

$$r = \sqrt{\frac{6\,DC}{A}}$$

where A is the oxygen consumption of the culture in ml/ml tissue/min. and D is the diffusion constant for oxygen through the culture. The average oxygen consumption of the cultures under conditions known to provide adequate oxygen supply has been found to be 1.37 μl/mg/hr (Table III) and this corresponds to $A = 0.023$. Krogh (22) found a value of $D = 1.4 \times 10^{-5}$ in frog muscle, but the relevance of this to rat lymph nodes seems doubtful, and as there appear to be no other data for biological tissue it was thought wiser to use the value $D = 1.98 \times 10^{-5}$ in 1 per cent KCl accurately determined by Carlson (5). Substituting these figures in the formula and assuming that the culture is surrounded on all sides by pure oxygen ($C = 1$), r becomes 0.7 mm. So even in the ideal case of a culture suspended in pure oxygen the radius of the culture would have to be < 0.7 mm if any oxygen at all is to reach the centre, and this corresponds to a limiting culture weight of 1.4 mg.

In the experiments already described the average weight of the cultures was about 1 mgm but cultures up to 1.5 mgm were frequently used and they always survived satisfactorily. It was found in some other experiments that when the culture weight reaches about 2 mgm some central necrosis appears. These experimental findings are therefore in general agreement with the theoretical predictions. Under the actual conditions of culture however, the base of the culture is in contact with the medium and not with pure oxygen, which would reduce the oxygen supply, but this is offset by the fact that the culture flattens somewhat on the wool and becomes ellipsoidal instead of spherical.

If the culture were in air instead of oxygen the theoretical limiting radius would be 0.32 mm and the weight 0.14 mg. Consider also the case of a culture separated from pure oxygen on all sides by a stationary layer of medium x cm thick. The oxygen now has to diffuse through the medium before it reaches the culture and the limiting radius, for zero oxygen concentration just being reached at the centre, is given by the following formula

$$r = 2\cos\frac{\theta}{3}\sqrt{\frac{2DC}{A} + x^2} - x, \text{ where } \cos\theta = \frac{-x^3}{\left(\frac{2DC}{A} + x^2\right)^{\frac{3}{2}}}$$

This formula was kindly derived for me by Dr. G. J. Neary of this Unit. Taking a random example, if $x = 1$ mm then r becomes 0.47 mm corresponding to a culture weight of 0.42 mg. This means that a 1 mm layer of stationary medium round the culture would reduce the permissible size of the culture by a factor of 3. These figures suggest that if air were used instead of oxygen or if the cultures were submerged in the medium, the oxygen supply would be insufficient to maintain cultures of the size normally used. Experimentally, this has been found to be the case. Quantitative data are available for only one experiment, details of which are given in Table IV. In other similar experiments the cultures were sectioned in order to study the distribution of pyknotic cells which were found mainly at the centre of the culture.

In chambers III and IV (Table IV) the culture conditions were (except for the pH) essentially the same as those in the method of Parker (33) and it is concluded that this method fails to maintain 1 mg lymph node cultures because the oxygen supply is inadequate. Parker (33) and Fagraeus (12) have reported the culture of small

TABLE IV

Pyknotic Counts of 24 hr. cultures in serum - glucose saline under Varying Conditions of Oxygen Supply.

Chamber	Position of cultures	Gas phase	No. of cultures	Mean Pyknotic Count
I	on cotton wool	O_2	8	2.28
II	»	Air	4	15.3
III	Cultures under 2 mm. depth of medium	O_2	8	14.0
IV	Cultures under 4 mm. depth of medium	O_2	8	22.3
V	»	Air	4	75.0

fragments of rabbit lymphoid tissue by this method but from their accounts it is not possible to judge how much lymphocyte degeneration occurred.

The oxygen supply could be improved in either of two ways: by supporting the cultures on cotton wool so that they project into the gas phase as is normally done here, or by stirring the medium by some rocking device as has been done by Medawar (28, 29). A roller tube would also serve the latter purpose and a few experiments were done on these lines. In one experiment five cultures of the usual type were suspended in 0.5 ml of the usual medium in a small roller tube filled with oxygen and after 48 hr. culture the average P. C. was 3.4. The cultures however shrank a good deal and a lot of lymphocytes were washed out as Medawar (29) found with his rocking method. Difficulty was experienced from the cultures sticking together and there was also the disadvantage that they could not be separately identified. These difficulties could no doubt be overcome, and there is no reason in principle why such a method should not give results equal to those obtained on cotton wool.

It has been found that cultures will survive 30 min. of almost complete anoxia provided they are at once returned to oxygen. This experiment was performed on 6 chambers with 4 cultures in each. After culture in oxygen for 24 hr., 60 ml of nitrogen was passed through the chamber. This has been shown by direct analysis of samples removed from the chamber to reduce the oxygen to < 0.17 per cent. After 30 min. in nitrogen, 60 ml of oxygen was passed and 6 hr. later the cultures were examined in the usual way. The average P. C. for the 24 cultures was 1.97 (S.E. 0.23), which is in the normal range.

2. *Alternatives to Cotton Wool.* The only purpose of the cotton wool is to hold the cultures at the surface of the medium so that they can get enough oxygen, and this can be done by other means. An obvious way is to place the cultures on the surface of a plasma clot, and such a method (the 'watch glass' technique) has been successfully used by Fell and others for the culture of a variety of embryonic organs (13). Lymph nodes have been cultured on plasma clot using the usual cups and chambers. In this case each cup contained 0.3 ml of heparinised cock plasma, 0.3 ml of rat serum and 0.05 ml of thrombin solution. The latter was made by dissolving 'Thrombin Topical' (Parke, Davis and Co.) in sterile distilled water and

TABLE V

Mean Differential Cell Counts of 48 hr. cultures on Various Semi-solid Media. Data presented as in Table I.

Medium	Gas phase	No. of cultures	Medium Lympho-cytes	Large Lympho-cytes	Pyknotic Lymphocytes		Lympho-cyte Mitoses
					Mean	S.E. of mean	
Cock plasma - rat serum - thrombin	O_2	14	1.81	0.26	2.77	0.77	
Cock plasma - thrombin	»	13	2.01	0.57	2.74	0.57	0.011
Fibrin foam - rat serum	»	10	0.22	0.06	5.56	1.36	
Serum - agar (+ glucose)	»	20	1.00	0.02	1.05	0.14	
»	»	16	0.74	0.02	2.68	0.30	
»	»	15	0.20	0	2.45	0.37	
»	»	24	0.87	0.01	2.57	0.33	
»	»	24	0.49	0.02	1.33	0.20	
»	»	8	0.58	0.04	3.44	1.03	
»	»	20	0.31	0.05	2.11	0.23	
»	»	24	0.39	0.05	1.97	0.23	
»	»	8	0.52	0	1.90	0.15	
»	3 p.c. CO_2 in O_2	13	0.25	0.03	1.85	0.45	
»	5 p.c. CO_2 in O_2	12	0.28	0.03	3.22	0.68	
Average of serum - agar cultures		184	0.54	0.03	2.11	0.11	0.003

Note. The results of 3 chambers (13 cultures) have been excluded from the serum-agar series, these were bad results and probably due to infection.

contained 20 N.I.H. units per ml. After coagulation of the medium the cultures were taken from serum and planted on the surface, leaving a very thin film of serum covering the coagulum. The results of one such experiment are shown in Table V and in this case the P. C. (2.77) was fairly satisfactory. In other experiments however the results were not quite so good and this was ascribed to the fact that the cultures liquefied the clot to a variable and sometimes great extent, so that they came to lie submerged in a pool of serum and did not get enough oxygen. Mammalian plasma was not tried because it is known to give a weak clot which is very readily liquefied by cultures. On pure cock plasma (clotted with thrombin) liquefaction occurred to about the same extent, but the experiment (Table V) is of interest in demonstrating a fairly good survival of the cultures on an entirely heterologous medium.

It is known that hanging-drop cultures of a variety of tissues liquefy cock-plasma clot only if mammalian serum is also present in the medium, and it has been postulated that a profibrinolysin present in mammalian serum is activated by a tissue kinase from the cultures (15). The liquefaction produced by the lymph-node cultures however cannot be explained on these lines because it occurred to the usual extent

even if the cultures were washed in saline and planted from saline on to the pure cock-plasma medium.

Fibrin foam soaked in serum was also tried. A piece of sterile fibrin foam (human) was cut aseptically into pieces 8 mm square and 5 mm deep. These were soaked in several changes of rat serum to get rid of citrate and gently squeezed to expel the air bubbles. A piece was placed in each cup, sufficient serum was added to keep the surface of the foam just wet and the cultures were then planted. The cultures liquefied the fibrin foam even more rapidly than ordinary plasma clot and the results were not satisfactory (Table V).

The fact that no glucose was added to the medium in these experiments is not thought to have influenced the results; evidence for this will be given in a later section.

In order to escape the problem of liquefaction it was decided to try a serum-agar jelly such as is used in bacteriology. A final agar concentration of 1.3 per cent was found to be most suitable; this gave a jelly which did not soften at 36° and which was slightly firmer than a cock-plasma clot. A stock solution of 4 per cent agar in 0.8 per cent NaCl was used, which was prepared as follows. 8 gm of Japanese agar fibre was cut into small pieces and washed in running tap water for two days and then in distilled water. The agar was drained and added to a flask containing 200 ml glass distilled water, 1.6 gm NaCl, 0.01 gm $NaHCO_3$ and 0.2 gm powdered charcoal. This mixture was then autoclaved at 10 lb for 5 min. in order to dissolve the agar and then immediately filtered through glass wool on a hot funnel. The purpose of the $NaHCO_3$ was to prevent hydrolysis of the agar at acid pH and the charcoal was intended to adsorb hypothetical toxic impurities. The solution was then reheated in the autoclave and at once filtered through filter-paper pulp on a Buchner funnel. The warm filtrate was poured into tubes which were subsequently sterilised in the autoclave. The culture medium was made by mixing 2 ml of the stock agar, previously melted at 100° C and then cooled to 60° C, with 4 ml of rat serum plus 0.1 ml of 10 per cent glucose, previously warmed to 60° C. Then 0.6 ml of the warm medium was pipetted into each cup where it set at once on cooling. The cultures were taken from serum and planted on the jelly. It was found that each culture at once produced a slight indentation of the agar surface and expressed a tiny pool of fluid which was just sufficient to keep the culture correctly wet; the intervening agar surface remained dry. This was entirely a mechanical effect due to the weight of the culture, because bits of wet cardboard of the same size and weight did the same thing; there was never any sign of digestion of agar by the cultures. The pH of the stock agar solution was 6.9 and that of the final culture medium 8.0.

This method has been described in some detail because the results obtained were quite satisfactory. Table V gives the results of 11 experiments performed in this way. The overall average P. C. for the 184 cultures was 2.11 (S.E. 0.11) which is to be compared with 1.61 (S.E. 0.08) for 282 cultures on cotton wool. As far as results go there is therefore little to choose between the two methods and the cotton wool method has been preferred chiefly on the grounds of convenience. The advantage of a removable fluid medium for biochemical analysis is obvious and there is the further advantage that the medium can be sucked off and replaced, or substances can be added to the medium during the course of the experiment, without having to transplant the cultures.

3. CO_2 *and* pH. The results listed in Tables I and III show that the lymphocytes

survive equally well whether the CO_2 is absorbed or not, and together with the last two experiments in Table V, that addition of 3 per cent or 5 per cent of CO_2 to the initial gas phase does not appreciably alter the results.

When the CO_2 was absorbed the initial pH of the medium was 8.6 falling to about 7.6 by the end of the culture period and the CO_2 concentration was zero throughout. When pure O_2 was used, without absorption of CO_2, the initial pH was 7.8 falling in some cases to 7.0 and the final CO_2 concentration in the gas phase (by direct analysis) varied between 5 and 10 per cent. With 3 per cent CO_2 in O_2 as initial gas phase the initial pH was 7.6 and with 5 per cent CO_2 it was 7.4. In the latter case the final pH was 6.5 and the final CO_2 about 15 per cent. It was observed that when the pH falls below about 7.2 the cultures, which are normally translucent, become white and opaque, though this does not harm the cells.

It appears therefore that lymphocytes under these conditions *in vitro* are not adversely affected by pHs ranging from 8.6 to 6.5 or by CO_2 concentrations in the gas phase ranging from 0 to 15 per cent. In view of the great sensitivity of lymphocytes to other adverse influences such as anoxia, hypertonic solutions, mitotic poisons, ionizing radiations, corticosterone, etc., this relative resistance to changes in pH and CO_2 is surprising.

4. *Culture Medium.* The fluid medium normally used in the cotton wool technique has been a 2 : 1 mixture of rat serum and glucose saline (0.7 per cent NaCl, 0.6 per cent glucose). Table VI shows experiments in which different culture media were used together with parallel control experiments with the normal medium.

Since protein and glucose were probably important variables in the medium it was desirable to have some information as to the normal levels of protein and glucose in the serum of the rats used in this work. Accordingly serum protein and serum glucose were determined in a series of rats of this colony, ranging in weight from 50 to 400 gm. The rats were not starved and blood was taken from the heart under ether anaesthesia as was done in the culture work. Total protein was determined by the specific gravity method using a pyknometer and the formula of Hoch and Marrack (17) which has been shown to be applicable to the rat (8). Glucose was determined by the method of King and Garner (21). Determinations were done in duplicate and the results are shown in Fig. 16.

Dealing first with the protein content of the medium, since the protein content of tissue fluid and peripheral lymph is only 1–2 per cent and this is the normal environment of the cells *in vivo*, it seemed possible that pure serum, with a protein content of 6–8 per cent might have a deleterious osmotic effect on the cells. Such however is not the case. Experiments 1 A, B (Table VI) show that the lymphocytes survive in pure serum (7.0 per cent protein) just as well as in serum-saline (4.7 per cent protein).

In tissue-culture work it is standard practice to use media in which the serum or plasma is diluted to at least 50 per cent with Tyrode or other saline. Undiluted serum is apparently never used, though so far as I know there is no experimental evidence that it is harmful to cells *in vitro*.

The only reason for deciding to dilute the serum somewhat with saline in the established technique used here, was that the saline then affords a convenient and easily sterilised vehicle for the addition of glucose and experimental substances to the medium. The theoretical reasons for choosing 0.7 per cent NaCl instead of the

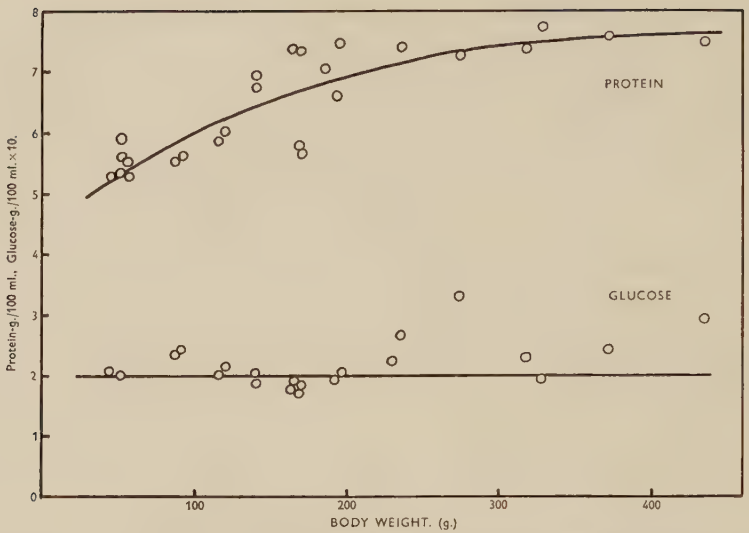


Fig. 16. Serum protein and serum glucose levels in rats of the Harwell colony. Blood taken from non-starved animals under ether anaesthesia.

usual Tyrode solution were as follows. In the first place it was decided to use a somewhat hypotonic solution because Tullis (39) studying the survival of blood lymphocytes in a 1:2 serum-saline mixture using various strengths of saline found optimal survival with a saline strength equivalent to 0.65 per cent NaCl. Bicarbonate was not added because this would have made the initial pH of the medium even more alkaline, and it was thought to err in this direction already, especially when the CO_2 was absorbed. Neither calcium nor phosphate was added because it is known that the concentration of inorganic phosphate, and probably also that of ionic calcium rises in serum during incubation at 36° C owing to the breakdown of phospholipids and calcium-protein complexes. Potassium was not specifically added because the batch of NaCl (A.R.) used to make the 0.7 per cent solution was found to contain sufficient potassium as impurity to provide the amount specified in the Tyrode formula. The reason for adding 0.6 per cent of glucose will be discussed later. Experiments 2 A, B in Table VI are parallel experiments using the same serum diluted in the one case with the glucose saline and in the other with Earle's (11) modification of Tyrode solution. The results are significantly different ($P = < 0.01$) and show a slight superiority of the glucose saline. But the difference is trivial; the chief advantage of the glucose saline is its simplicity and ease of sterilisation.

The normal medium contains 66 per cent of serum, and it was of interest to try further dilution for which purpose it seemed wiser to use Tyrode. The results obtained in three parallel experiments in which the same serum was used and diluted with Earle's Tyrode to 66 per cent, 50 per cent and 33 per cent respectively are shown in Table VI (3 A, B, C). In 50 per cent serum the lymphocyte survival was not quite so good as in 66 per cent and in 33 per cent it was much worse. For optimal survival, therefore, the medium should contain at least 50 per cent of serum, which

TABLE VI

Pyknotic Counts of 48 hr. Cultures on Cotton Wool in Various Fluid Media.
Gas phase O_2, CO_2 not absorbed.

Brackets enclose parallel experiments using litter-mate animals and the same batch of serum.

Exp. No.	Fluid Medium	No. of cultures per cup	Total No. of cultures	Pyknotic Count	
				Mean	S.E. of mean
1 A	Serum - saline, 2 : 1	4–5	13	1.27	0.33
1 B	Serum	4–5	9	1.56	0.25
2 A	Serum - glucose saline, 2 : 1	5–6	11	1.27	0.16
2 B	Serum - Tyrode, 2 : 1	5–7	27	2.16	0.27
2 C	Heparinised plasma - glucose saline, 2 : 1	5–6	11	3.13	0.54
3A	Serum - Tyrode, 2 : 1	7–8	15	2.28	0.23
3B	Serum - Tyrode, 1 : 1	7–8	16	2.75	0.44
3C	Serum - Tyrode, 1 : 2	7–8	14	6.60	0.73
4	Horse serum - glucose saline, 2 : 1	5–6	23	5.63	0.77
5	Rabbit serum - glucose saline, 2 : 1	5–6	17	3.15	0.40
6	Serum - saline, 2 : 1	4–5	13	1.27	0.33
[1]7	»	6–7	37	2.84	0.29
8	»	6–7	19	1.52	0.15
9	»	8–11	28	1.30	0.14
10	»	14	14	2.20	0.37
11	»	5–10	47	10.96	0.98
12	»	8–9	34	6.64	1.17
13 A	Serum - saline, 2 : 1	9	9	22.91	4.50
13 B	Serum - glucose saline, 2 : 1	8–9	26	1.65	0.26

means about 3.5 per cent of protein. The limiting factor in these experiments might, of course, have been the concentration of some serum constituent other than the protein, but not glucose or the ordinary inorganic ions.

In Experiments 2 A, C (Table VI) heparinised rat plasma (1 mg (160 I.U.) heparin/ml) was compared with serum from the same animal, both being diluted with glucose saline. The results are significantly different ($P = < 0.01$) and in favour of the serum. Citrated plasma was tried but this killed all the cells in a few hours. Horse serum (Exp. 4) and rabbit serum (Exp. 5) were found to be inferior to the usual rat serum.

In a number of the normal culture experiments listed in Tables I, III and V the serum was quite milky owing to the presence of small fat droplets (chylomicrons), while in other cases the serum was clear. This difference in the serum was not correlated with any differences in lymphocyte survival or metabolism. It seemed clear that the presence of quite a large amount of fat in the serum did no harm and this

[1] Lymph nodes from Compton rats.

is of interest because it has been suggested that fat may be toxic to lymphocytes (3). When cultures were grown on serum-agar and a milky serum was used, it was observed that under and around each culture the medium became quite clear, indicating a disappearance of the fat. This clear zone gradually spread during the period of cultivation and after 48 hr. it extended about 1 mm beyond the edge of the culture. Since no cells migrate into the agar the most likely explanation is that a lipase diffuses out from the culture. The fact that these cultures locally liquify a pure cock-plasma clot in the absence of mammalian serum suggests that a proteolytic enzyme also diffuses out. Lymphocytes have been shown to contain both lipase and cathepsin (2) and it is tempting to suggest that they may secrete such enzymes, but inasmuch as a few degenerating cells are always present it is possible that the enzymes are simply liberated during cell breakdown.

The remaining variable to be considered is glucose. Table III shows that even in the presence of a large excess of glucose, the glucose consumption varies considerably from experiment to experiment. It is therefore not possible to define at all closely the minimum amount of glucose which the medium must contain in order to support the metabolism of a standard number of cultures for 48 hr. But a few generalisations may be made. The serum used in these experiments was usually taken from rats weighing 150 to 200 gm and from the data given in Fig. 16 it may be assumed that it contained about 0.2 per cent of glucose. Taking this figure of 0.2 per cent, if undiluted serum were used as the medium, the total amount of glucose present in the 0.7 ml used in each cup would be 1.4 mg. Reference to the 'glucose consumed' column of Table III shows that in only two instances, and then but slightly, did the glucose actually consumed exceed 1.4 mg. So in an average experiment with undiluted serum as medium there should be no need to add any extra glucose. The successful cultures in pure serum reported in Table VI confirm that this is the case.

If, however, the serum were diluted with 0.7 per cent NaCl (without glucose) in the usual 2:1 proportion, the total amount of glucose available would only be 0.93 mg. Reference to Table III shows that in 10 out of the 25 cases the glucose consumed was more than 0.93 mg. In such a medium therefore the glucose concentration is borderline and might in many cases be insufficient to maintain the cultures. This medium has been tried out in seven experiments (Table VI). Expts. 6–10, performed in March–July 1950, showed apparently that the serum-saline was a satisfactory medium; there was no sign that the cultures suffered from lack of glucose even with 14 cultures in one cup. On the other hand Expts. 11 and 12, performed in December 1950, seemed to show that such a medium was inadequate, and a further experiment (13 A, B) performed at this time proved that the cultures were in fact failing from lack of glucose. The discrepancy between these two sets of results can perhaps be accounted for by the fact that in Expts. 6–10 the serum was obtained from rats larger than those usually used (250–350 gm). Fig. 16 shows that in these larger rats the serum glucose is frequently higher than 0.2 per cent and may even exceed 0.3 per cent, a fact which was not known at the time the experiments were made.

The conclusion is that serum-saline cannot be relied upon to maintain the cultures and it is therefore wiser to add an excess of glucose in all cases. This is conveniently done by adding 0.6 per cent of glucose to the 0.7 per cent NaCl, so that the initial glucose concentration in the medium is about 0.4 per cent. The excess does no harm and is in fact sufficient to maintain the cultures for four days if necessary.

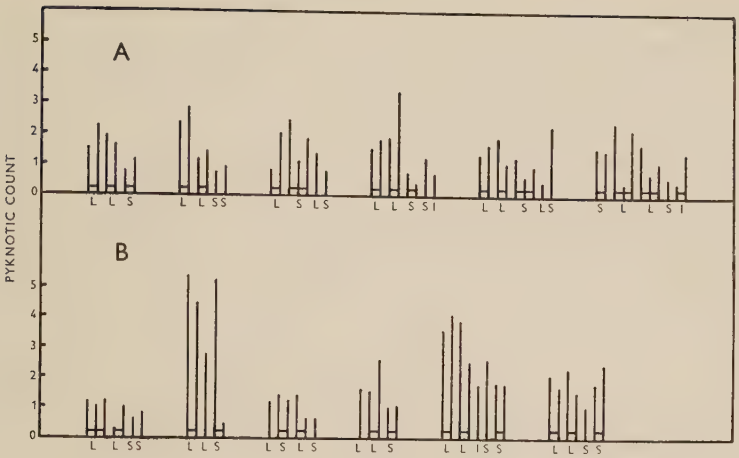


Fig. 17. Pyknotic counts of individual cultures in two typical experiments, A and B. The grouping corresponds to the six culture chambers used in each experiment. L, S, I indicate lumbar, sacral and iliac nodes respectively. Where a node was divided into two or three portions, the corresponding sister cultures are indicated by horizontal bridging.

5. *Animal Variation.* In most experiments six culture chambers were set up in parallel fashion with the same culture medium, and each chamber contained all the cultures derived from one individual animal. The serum was obtained from a different rat (or rats) in each experiment, so any significant animal variation in the serum would be reflected in differences between the results of the individual experiments. On the other hand, variation among the six litter-mate animals from which the cultures are derived would be reflected in chamber to chamber variation within the experiment.

With regard to variation of the serum, the results listed in Tables I and V show slight differences in the pyknotic count between one experiment and another. These differences, though small, are in many cases statistically significant ($P = < 0.01$). There is no proof that they are due to the serum; they might equally well be due to differences between litters. The point is rather that any important variation of the serum in respect of lymphocyte maintenance would show up on these figures and the conclusion is that such variation amounts at the most to very little. The age or sex of the animal which provided the serum did not influence the results.

In some experiments rats from a different colony were used. They were taken from the Wistar colony of the A.R.C. Field Station, Compton, Berkshire. These rats differed from the Harwell rats in that they were fed a wet-mash diet of natural food stuffs and housed at ordinary room temperature.

In the experiment indicated in Table I, lymph nodes from Harwell rats were cultured in the serum of a Compton rat, while in experiment 7, Table VI, lymph nodes from Compton rats were cultured in serum of Harwell rats. The results again indicate that the serum is an unimportant variable.

A significant chamber to chamber variation was found in only a few of the experiments. Detailed statistics have not been worked out, but Fig. 17 B shows graphically a case in which such a variation was obvious, while Fig. 17 A shows an example of the more common case in which it was not. The differences between cultures derived from different animals are therefore usually negligible and at most slight. Fig. 17 also illustrates the degree of culture to culture variation which is usually found.

6. *Note on Reticular cells (Macrophages).* The term reticular cell is used here to include both the fixed cells of the reticulum and the free ameboid macrophages. These cells were present in the films of all the cultures, but in very varying numbers (0.1–2.0 per cent of total cells). They were always carefully examined as it was anticipated that under some conditions they might, like the lymphocytes, show signs of degeneration or death. It is a striking fact that no dead or degenerating reticular cells were ever seen in the normal cultures, even in 8 day cultures, nor were they ever found under any of the abnormal conditions described in this section—except in the case of the citrated plasma: citrate killed the reticular cells as well as the lymphocytes. This means that the reticular cells survived certain environmental conditions which the lymphocytes were unable to tolerate, namely, low oxygen tension, low glucose concentration, dilution of serum to 33 per cent, heterologous serum. The only abnormality encountered in the reticular cells was that in the serum-agar cultures their cytoplasm was often greatly distended with vacuoles.

DISCUSSION

Lymph-node lymphocytes are the cells of the body most sensitive to the destructive action of ionizing radiations (4) and nitrogen mustard (20); and they are rather specifically destroyed by adrenal cortical hormone (9) and a great variety of mitotic poisons (10). It has been shown above that these cells are unable to survive some environmental conditions which are well tolerated by reticular cells, and which would certainly be tolerated by fibroblasts. These observations suggest that lymphocytes are among the most delicate cells of the body, and it is not surprising that they are so difficult to maintain *in vitro*. This frailty might be in some way due to their relative paucity in cytoplasm.

The survival of the great majority of the lymphocytes in these lymph-node cultures may be contrasted with the early death of a large proportion of the cells in hanging-drop cultures of lymphoid tissue and in cultures of lymphocyte suspensions. It is important to know why this should be, but as yet no very satisfactory answer can be given. I have previously shown (38) that the death of lymphocytes in hanging-drop cultures is not due to lack of oxygen, glucose or any other serum constituent.

It has also been found that a suspension of lymphocytes fails to survive under what would appear to be optimal cultural conditions. In this experi-

ment a small drop of a lymphocyte suspension, made by cutting up a lymph node in serum, was cultured on the surface of serum-agar in oxygen, and over 90 per cent of the cells were dead after 8 hr. The explanation must therefore be sought in some biological difference between the undisturbed lymphocytes in a lymph node and the free scattered lymphocytes of hanging-drop cultures or cell suspensions.

One obvious factor is trauma. It has already been shown that when a lymph node is cut across, cells adjacent to the cut edge die during the succeeding two hours. In the preparation of hanging-drop cultures and lymphocyte suspensions a lymph node is cut up into many small pieces and it is probable that a great number of the cells are damaged in this way, which could account for their early death in the cultures.

Another difference is that in the intact lymph node the lymphocytes maintain a close anatomical relationship with the reticular cells from which they have been derived. It is known from tracer studies that lymphocytes have a high rate of turnover of both proteins and nucleic acids. A continual synthesis of protein and nucleic acid is therefore required for maintenance. Since lymphocytes have relatively much less cytoplasm than any other somatic cell it is possible that they do not possess a complete enzymic equipment and that they have to rely on the parent reticular cells for some step in protein or nucleic acid synthesis.

One of the main objects of tissue-culture technique is to provide the cells with an environment as similar as possible to that enjoyed *in vivo*. It is generally assumed that this is best done by using a medium in which the constituents are all present at 'physiological' concentration. This may be true in respect of the outgrowth zone of an ordinary tissue-culture, but if the objective is a total survival of the explant, the situation is more complicated. The total survival of a tissue fragment, as distinct from a mere peripheral survival in the form of an 'outgrowth,' is conditional on the supply of oxygen and foodstuffs and the removal of waste products being adequately maintained by diffusion. There will be a concentration gradient from the centre of the culture to the periphery in respect of all substances consumed or excreted by the cells, so the environment of the cells at the centre will be very different from that provided by the external medium. In order to facilitate this diffusion and so improve conditions at the centre of the culture it would seem better to use a medium in which the concentrations of substances consumed by the cells (e.g. O_2, glucose) are higher than physiological, while the concentrations of substances excreted by the cells (e.g. CO_2, H ions) are lower than physiological. The case of oxygen has

been fully considered in this paper and a similar situation must exist for other components. For example, it is probably better to use no CO_2 in the gas phase and a pH of 8 or more, rather than the 5 per cent CO_2 and pH 7.4 which is usually aimed at. This principle has been employed in the work described in this paper and it is suggested that it may be a general principle of some importance in tissue culture technique.

This method of lymph-node culture and cell counting can be used for accurate quantitative measurement of the lethal effect of ionizing radiations, toxic substances, etc., on lymphocytes under controlled environmental conditions *in vitro*.

SUMMARY

Lymph nodes from young rats have been successfully cultured on the surface of cotton wool soaked in serum-saline, in an atmosphere of oxygen. An alternative method of culture on serum-agar is also described.

The health of the cultures was assessed by determining the percentage of the lymphocytes which were pyknotic (dead). This 'pyknotic count' was made on wet-fixed film preparations of the cultures. The cultures regularly survived for four days, at which time the average pyknotic count was always less than 2 per cent. The lymph nodes used exhibited very little lymphopoiesis *in vivo* and still less *in vitro*. The main object of the method was firstly to maintain, and thereafter to experiment on, a healthy population of lymphocytes *in vitro*.

It was shown that the lymphocytes would only survive under conditions of good oxygen supply, which meant in practice that the greater part of the surface of the culture had to be in contact with pure oxygen gas. The lymphocytes were not adversely affected by pHs ranging from 8.6 to 6.5, or by CO_2 concentrations in the gas phase ranging from 0 to 15 per cent. Various fluid culture media were tried and the most satisfactory proved to be a 2:1 mixture of rat serum and a solution containing 0.7 per cent NaCl and 0.6 per cent glucose. It was concluded that lymphocytes are more exacting in their cultural requirements than are macrophages or fibroblasts.

Direct measurements of the oxygen and glucose consumptions of the cultures were made from which it appeared that a normal level of carbohydrate metabolism was maintained *in vitro*. Evidence was obtained that proteolytic and lipolytic enzymes diffused from the cultures into the medium.

It is a pleasure to thank my technical assistants Mr. W. R. Lush and Miss P. E. Law for their unremitting care and attention to detail, to which, such success as

we have achieved, is largely due. I am grateful to Dr. G. J. Neary of this Unit for his help with the mathematical treatment of oxygen diffusion and to Miss M. E. MacKay of the Lister Institute for a supply of fibrin foam.

REFERENCES

1. Andreasen, E., and Christensen, S., *Anat. Record*, **103**, 401 (1949).
2. Barnes, J. M., *Brit. J. Exptl. Path.*, **21**, 264 (1940).
3. Bloom, W., *Arch. exp. Zellforsch. Gewebezücht.*, **5**, 269 (1928).
4. —— Histopathology of Irradiation. New York, McGraw-Hill, p. 751, 1948.
5. Carlson, T., *J. Am. Chem. Soc.*, **33**, 1027 (1911).
6. Colowick, S. P., Cori, G. T., and Slein, M. W., *J. Biol. Chem.*, **168**, 583 (1947).
7. De Bruyn, P. P. H., *Anat. Rec.*, **93**, 295 (1945).
8. Dicker, S. E., *J. Physiol.*, **107**, 11 P (1948).
9. Dougherty, T. F., and White, A., *Am. J. Anat.*, **77**, 81 (1945).
10. Dustin, P., *Nature*, **159**, 794 (1947).
11. Earle, W. R., *J. Natl. Cancer Inst.*, **4**, 165 (1943).
12. Fagraeus, A., *Acta Med. Scand., Suppl.* **204** (1948).
13. Fell, H. B., *J. Roy. Microscop. Soc.*, **60**, 95 (1940).
14. Gerard, R. W., *Biol. Bull.*, **60**, 245 (1931).
15. Goldhaber, P., Cornman, I., and Ormsbee, R. A., *Proc. Soc. Exptl. Biol. Med.*, **66**, 590 (1947).
16. Hall, V. E., and Furth, J., *Cancer Research*, **2**, 411 (1942).
17. Hoch, H., and Marrack, J., *Brit. Med. J.*, **2**, 151 (1945).
18. Jackson, H., Jr., Parker, F., Jr., and Glover, E. C., *J. Exptl. Med.*, **52**, 547 (1930).
19. Kindred, J. E., *Am. J. Anat.*, **67**, 99 (1940).
20. —— *Arch. Path.*, **43**, 253 (1947).
21. King, E. J., and Garner, R. J., *J. Clin. Path.*, **1**, 30 (1947).
22. Krogh, A., *J. Physiol.*, **52**, 391 (1919).
23. Laznitski, A., and Woodhouse, D. L., *J. Anat.*, **78**, 121 (1944).
24. Macmillan, R. E., *Anat. Record*, **39**, 155 (1928).
25. Maximow, A., *Arch. mikroskop. Anat. Entwicklungsmech.*, **97**, 283 (1923).
26. —— *Arch. exp. Zellforsch. Gewebezücht.*, **5**, 169 (1928).
27. Mayer, P., *Z. wiss. Mikroskop.*, **20**, 409 (1903).
28. Medawar, P. B., *Quart. J. Microscop. Sci.*, **89**, 187 (1948).
29. —— *ibid.*, **89**, 239 (1948).
30. Murray, R. G., *Am. J. Anat.*, **81**, 369 (1947).
31. Parker, R. C., *Science*, **83**, 579 (1936).
32. —— *ibid.*, **85**, 292 (1937).
33. —— Methods of Tissue Culture. New York, Paul B. Hoeber, p. 142, 1938.
34. Redfield, R. R., and Barron, E. S. G., *Arch. Biochem.*, **26**, 275 (1950).
35. Rekers, P. E., U. S. Atomic Energy Commission, declassified document, MDDC 1763, 1948.
36. Schrek, R., *Endocrinology*, **45**, 317 (1949).
37. Strangeways, T. S. P., and Fell, H. B., *Proc. Roy. Soc. London*, B, **99**, 340 (1926)
38. Trowell, O. A., *J. Physiol.*, **110**, 4 P (1949).
39. Tullis, J. L., *Am. J. Physiol.*, **148**, 708 (1947).
40. Victor, J., *ibid.*, **111**, 477 (1935).
41. Victor, J., and Potter, J. S., *Brit. J. Exptl. Path.*, **16**, 243 (1935).
42. Warburg, O., Posener, K., and Negelein, E., *Biochem. Z.*, **152**, 309 (1924).
43. White, J. C., *J. Path. Bact.*, **59**, 223 (1947).

THE EFFECT OF PURINES AND RELATED SUBSTANCES UPON CELLS IN CHICK TISSUE CULTURES

ARTHUR HUGHES

Strangeways Research Laboratory, Cambridge, Great Britain

Received May 15, 1951

Ciné-microscopy by phase contrast makes it possible to observe in detail the effects on an agent on living cells in tissue culture. In such studies on cells in mitosis it is possible to select a dividing cell in the outgrowth of a culture and to add a substance in solution while the cell is under observation. In this way the effects of a number of agents on mitosis have been examined (9, 10, 11).

In chick cells, and probably in many others, the development and function of the mitotic spindle are the most sensitive features of the mitotic process. Some substances also affect the dividing cell at other points; but generally metaphase is sensitive as well. When a chick cell is inhibited in metaphase by colchicine, for example, it remains in this condition without further change. The chromosomes stay in their metaphase condition. It has been recently observed that the antifolic acid drug 4 aminopteroyl glutamic acid (aminopterin) acts as a metaphase inhibitor (12). Most cells remain in arrested metaphase, but occasionally the whole metaphase group of chromosomes has been seen to enter reconstruction and form one nucleus, which is presumably tetraploid (10).

This observation suggested that it might be worth examining the effects of competitive antagonists of other natural substances on dividing cells. The purine antagonist benzimidazole (17) was then found to give the same result as aminopterin but much more regularly. However, when attempts were made to counteract the effect by the addition of purines, it became clear that these also acted in the same way and that the action of benzimidazole on cells in cultures is therefore not an example of competitive antagonism. In all probability benzimidazole, like the purines themselves, acts directly upon the living cell. It was then decided to investigate in detail the action on dividing cells of purines and compounds related to them.

METHODS

The methods of tissue culture and of ciné-micrography used in this work have already been described (8), and their application to the study of the effects of chemical substances upon the dividing cell (9, 10).

The compounds used in the present work were mostly commercial preparations. Some specimens were presented by various donors to whom I am greatly indebted for these gifts:

Professor H. N. Green (Sheffield); pure adenosine, inosine, and adenine-9-glucoside; Professor A. R. Todd; adenine-9-xyloside; Dr K. Bailey (Cambridge); Ba salt of A.T.P; Dr Overend (Birmingham); cytosine, ribose, and many other substances; Dr Hitchings (Wellcome Laboratories, U.S.A.); guanazolo and diaminopurine; Dr Roblin (Lederle Laboratories, U.S.A.); guanazolo; Dr Goodman (Colorado); Cenzimidazole N-glucoside.

The substances were dissolved in Tyrode saline at 37° and where necessary the pH was adjusted to within the range 7.4–7.7. The compound under test at various dilutions was then added to the culture for periods of 30–60 minutes, during which a photographic record of a cell in the culture was made. As soon as this was completed, the culture was fixed, and after staining the same cell was identified in the permanent preparation, and compared with its appearance during life. Usually four cultures were treated with each dilution of the substance. There were two sets of control cultures in addition; one was treated with Tyrode saline for the experimental period and the other was fixed without treatment. The stains used were either Ehrlich's haematoxylin of May-Grünwald-Giemsa, which Jacobson and Webb (12) have recently introduced for the study of tissue cultures.

I am greatly indebted to Mr L. J. King and to Miss J. Rawlinson for providing the tissue cultures used in the course of this work, and to Miss Rawlinson for staining all the cultures.

From the Ba salt of A.T.P., the nucleotide itself was prepared. The original substance was dissolved in N/HCl and the calculated quantity of sodium sulphate added to precipitate the barium as sulphate, which was centrifuged off. The supernatant was brought to pH 7.6, and the necessary salts added to convert the solution to Tyrode saline. The resulting concentration of A.T.P., apart from its decomposition during the experiment, was calculated from a phosphate estimation after complete hydrolysis which Dr Webb kindly undertook.

RESULTS

One or more of the following effects may be seen in tissue cultures treated with the substances which have been used in this work (Table I).

[1] *Pre-telophase reconstruction in dividing cells.* Mitosis proceeds normally until the end of metaphase, but anaphase is inhibited to a varying extent and cleavage of the cell is prevented. Reconstruction of the chromosomes, however, takes place at the normal rate. If anaphase is wholly or partly

suppressed, all the chromosomes on the metaphase plate reconstruct as one nucleus which is presumably tetraploid (Fig. 1–5). Often, anaphase movement ceases before the chromosomes have moved more than half their normal distance apart, and both groups fuse together when reconstruction begins (Figs. 6–9; 10–14). Here again, one nucleus is formed. Two nuclei result when the distance between the daughter groups is too great to permit of their fusion.

Adenine, 2–6 diaminopurine and benzimidazole all exert this effect on cells already in metaphase. A few minutes after addition of these substances, such cells have been followed through 'metaphase reconstruction.' When cultures have been treated for 30–60 minutes with these substances and then fixed, a few cells can usually be found in which there is either one large telophase nucleus, sometimes of irregular shape, or with two early interphase nuclei within one cell. With increasing dilution of these agents, the number of cells which undergo pre-telophase reconstruction decreases, and normal telophases also are found in the outgrowth of the cultures. To estimate the effectiveness of each compound in suppressing cleavage, cultures in batches of four were treated with a series of dilutions and the minimum concentration estimated at which no normal telophases were found in the outgrowth; for both adenine and diaminopure this was found to be about 3.5 m Molar, while 8.5 m Molar benzimidazole was necessary to achieve the same effect. After treatment of a culture with a saturated solution of xanthine in Tyrode at 37° C (about 6 m Molar), normal telophases were still present in the outgrowth but also one or two binucleate cells, which had undergone nuclear reconstruction in late anaphase, were seen.

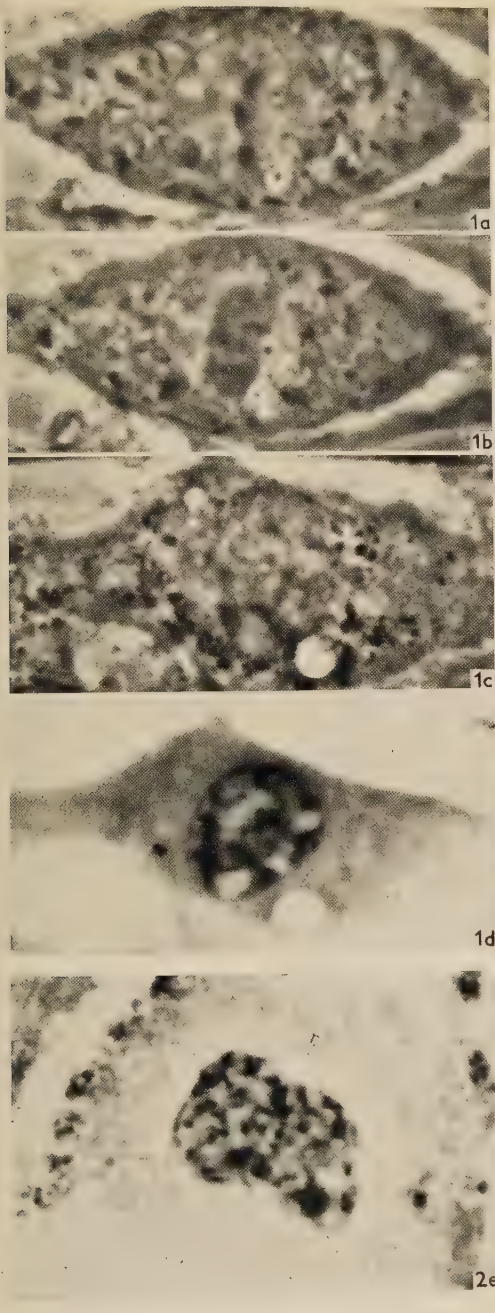
Cytosine, the pyrimidine corresponding to adenine, shows a slight adenine-like effect, for in cultures treated therewith some cells are found which have undergone pre-telophase reconstruction, though normal telophases are

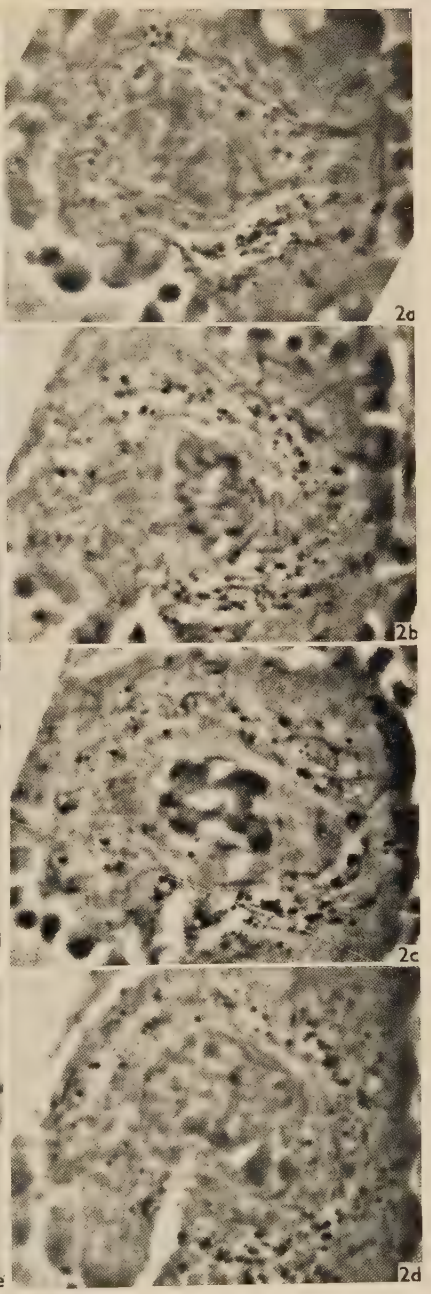
---

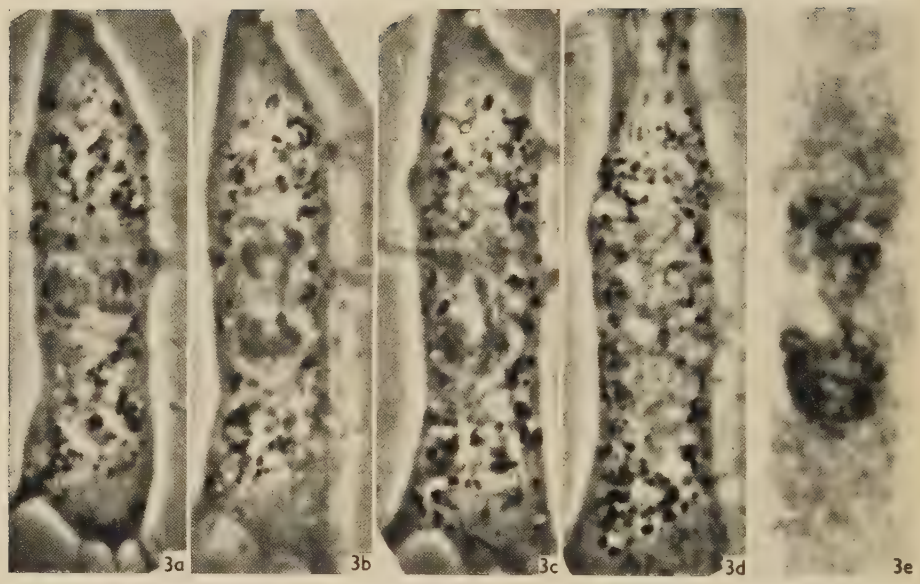
PLATE I.

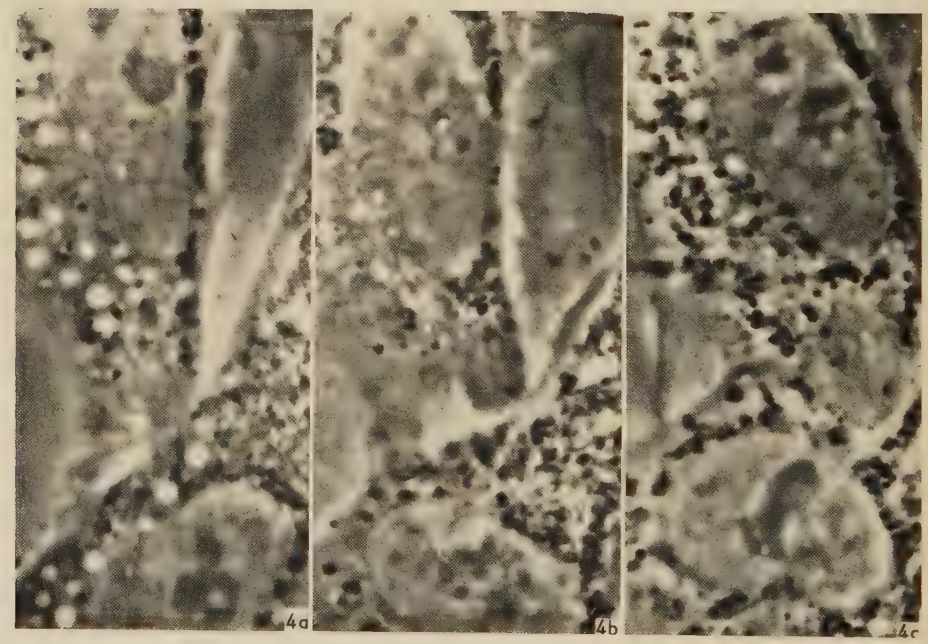
Fig. 1 (a–d). Effect of 8.5 m Molar benzimidazole on cell in metaphase in chick culture:
(a) 2 minutes after addition of agent—clear metaphase plate.
(b) 7 minutes after (a). Metaphase reconstruction beginning.
(c) 44 minutes after (1). Reconstruction complete.
(d) Fixation (Zenker). 50 minutes after (a). Stained in Ehrlich's haematoxylin.

Fig. 2 (a–e). Effect of 5.8 m Molar 2.6-diaminopurine on cell in metaphase in chick culture:
(a) 2 minutes after addition of agent.
(b) 3 minutes after (a). Anaphase begins.
(c) 6 minutes after (a). Anaphase has stopped.
(d) 34 minutes after (a). One nucleus has been formed from both groups of chromosomes.
(e) Fixation (methanol). 37 minutes after (a). Stained in May-Grünwald-Giemsa.


1a
1b
1c
1d
2e


2a
2b
2c
2d


3a
3b
3c
3d
3e
10 μ


4a
4b
4c

much more numerous. The cleavage-inhibiting effect of xanthine and cytosine is of the same order as that of aminopterin, which has been described in a previous paper (10).

The effect of guanine could not be tested owing to the low solubility of this compound.

In cultures treated with purines, normal prophases and metaphases are seen in the outgrowth, but also some arrested metaphases may be found in which the chromosomes are scattered, and no spindle is present. Thus these substances can inhibit cells in metaphase. Such cells probably remain in this condition until they degenerate. It is probable that cells which enter prophase in the presence of these substances tend to undergo metaphase arrest, while cells already in metaphase when the substance is added are inhibited at a later stage of mitosis and cleavage of the cell is prevented. Since even here the anaphase movement of the chromosomes is curtailed, it is clear that also under these circumstances the spindle is affected, though to a lesser degree than where total inhibition occurs in metaphase.

Nucleosides and nucleotides do not generally inhibit cleavage, although occasionally a cell which has undergone pre-telophase reconstruction will be found. They exert other effects on cells in cultures, which will now be described.

[2] *Pre-prophase inhibition.* When cultures are treated with adenosine, or its nucleotides, the number of cells in prophase in the outgrowth is reduced. This effect has been studied in fixed preparations by comparing batches of four cultures treated for 45–50 minutes with one concentration of a substance with another batch either treated with Tyrode saline or left untouched.

PLATE II.

Fig. 3 (a–e). Effect of 7.4 m Molar adenine on a cell in metaphase in chick culture:
(a) 1 minute after addition of agent.
(b) 10 minutes after (a). Cell in anaphase.
(c) 18 minutes after (a). Anaphase movement has stopped and nuclear reconstruction has begun.
(d) 30 minutes after (a).
(e) Fixation (methanol). 35 minutes after (a). Stained in May-Grünwald-Giemsa.
The two daughter nuclei are joined by a bridge formed from the lagging chromosome(s) seen in (b).

Fig. 4 (a–c). Reversible effect of 7 m Molar adenosine on the nucleoli of interphase cells in chick culture:
(a) Two interphase cells 2 minutes after addition of agent. This was withdrawn 22 minutes after (a) when the nucleoli had begun to fragment. The culture was washed several times with Tyrode and photography was resumed.
(b) 35 minutes after (a). The nucleoli are fragmented.
(c) 157 minutes after (a). The nucleoli have largely recovered their coherence.

The number of prophases in cultures is variable; Tyrode does not appreciably reduce their number in this period. From the results obtained, the concentration just sufficient entirely to abolish prophases has been judged, although owing to the variation between individual cultures, no very precise measurement of this is possible. If cultures are treated for one hour with a concentration of adenosine sufficient to prevent the entry of cells into prophase, and the solution is then withdrawn, it will be seen that after a further hour, prophases reappear in the outgrowth.

[3] *Nucleolar fragmentation in intermitotic cells.* When adenosine in Tyrode saline is added to a culture, during the following 10–15 minutes the nucleoli of many intermitotic cells break up into separate granules. The extent to which this occurs varies from cell to cell, and may reach the extreme state when the nucleolar material is reduced to a number of scattered granules within the nucleus. These bear no apparent relation to the Feulgen-positive granules in the normal interphase nucleus, each of which probably represents the heterochromatic regions of a number of chromosomes as in mouse cells (5).

In cultures treated with nucleosides and nucleotides, or with benzimidazole, a moderate degree of nucleolar fragmentation can readily be recognised in the stained preparations, particularly where a basic dye is used to reveal ribonucleic acid, as with the May-Grünwald-Giemsa stain, the use of which for this purpose has been described by Jacobson and Webb (12). Time-lapse films of the living cell, however, can reveal an even smaller degree of this effect, in which the nucleolus does not break up, but slowly changes in form. Normally no movement is seen within the nucleus of the mature intermitotic cell, nor is there any change in shape of the nucleoli when followed for some hours. This is in contrast to the early post-telophase nucleus, as is described by Fell and Hughes (5) for the mouse. Although as already stated nucleosides and nucleotides can prevent cells from entering prophase they do not generally inhibit a mitosis which has already begun. Anaphase and cleavage proceed normally and chromosome groups reconstruct into daughter nuclei. In cultures fixed after one hour's treatment with adenosine and stained with May-Grünwald-Giemsa, it can be seen that in the early interphase nuclei, the blue staining material is abnormally scanty, and is sometimes restricted to a few granules. On the other hand, the chromosomes themselves are abnormally distinct in such nuclei. The process of nuclear reconstruction is therefore retarded by adenosine and the nucleolar material of both telophase and intermitotic nuclei is diminished.

The fragmentation of nucleoli by adenosine and benzimidazole is rever-

TABLE I

Action	Substance	Minimum effective concentration in m Mols	Action
1. Pre-telophase reconstruction	Adenine	3.7	No normal telophases
	2–6 Diaminopurine	3.4	Normal telophases rare
	Benzimidazole	8.5	No normal telophases
	Xanthine	6.6	Occasional anaphase reconstructions
	Cytosine	9.0	Occasional anaphase reconstructions
2. Pre-prophase inhibition	Adenosine Adenylic acids ATP	0.6–1.2	Prophases none, or very rare
3. Nucleolar fragmentation	Adenosine	0.4	Nucleolar granular
	Adenylic acids	1.2–2.9	Partial break-up
	ATP	2.5	Only very slight
	Guanosine	4.3	Some nucleoli granular
	Benzimidazole	4.3	Nucleoli granular
4. Inactive under (1–3)	Benzimidazole –N. glucoside	3.6	
	Adenine-9-glucoside	2.5	
	Adenine-9-xyloside	3.5	
	Guanozolo (8-azaguanine)	6.7	
	Inosine	2.7	
	Xanthosine	1.7	

sible. After treatment of cultures with either of these substances for a sufficient period for the nucleoli of most cells to fragment, addition of fresh Tyrode, after repeated washing with saline, will result in the regeneration of the nucleoli in 30–40 minutes (Fig. 4). This also occurs more slowly if the adenosine or benzimidazole saline is merely removed from the culture.

The pure adenosine supplied by the kindness of Professor Green was found to be more effective at the same dilution in fragmenting nucleoli than was a commercial sample which was found by Dr. Webb to contain about 1 per cent of ash.

Generally effects [2] and [3] are given by the same substances, but only adenosine is equally effective for both. As pre-prophase inhibitors, nucleotides have the same potency as adenosine but have much less effect on nucleoli. On the other hand, benzimidazole and guanosine break up nucleoli, but do not prevent every cell from entering prophase. Thus in cultures treated

with these two substances it can be shown that occasional cells in which the nucleoli are broken up are still able to enter mitosis. Furthermore, with guanosine, mitosis is completed almost normally, except for the smallness of the nucleoli in the daughter cells.

No signs of any general toxic effects on cultures treated with these active substances were seen under the conditions of these experiments.

[4] *Substances without these effects.* In Table I is given a list of purine derivatives and analogues which were found to be without any of the three effects which have just been described. They also were found not to exert any general toxic effects on the cultures within a period of one or two hours.

Some experiments were made with two amino-acids in an attempt to see how far these actions on dividing cells were correlated with the presence of amino groups in biological molecules. Glycine and histidine were applied to cultures at concentrations of 25–50 m Molar under standard conditions. Cultures treated with glycine become unhealthy within a few hours, but toxic symptoms were less marked with histidine. Mitoses followed in cultures immediately after treatment with glycine were much distorted. Several nearly normal divisions were followed in cultures to which histidine had been added. In neither series were signs of any specific effects to be seen.

DISCUSSION

Of the three effects on cells in culture which have just been described, the disintegration of nucleoli is the only one which may possibly be specific, for no substances other than those listed under heading [3] in Table I are yet known to break up nucleoli under those conditions. The first effect, the suppression of cell division without complete inhibition of the nuclear cycle, is occasionally provoked by aminopterin and a nitrogen mustard (10). Rosenfeld (14) observed that ether could produce this effect in tissue cultures; sea urchin eggs were occasionally found to respond in this way to the same treatment by Wilson in 1902 (16).

The inhibition of cells in culture from entry into prophase is also unspecific. Fluoride, iodoacetate (1), hypotonic saline and isotonic sucrose (Hughes, unpublished) are as rapidly effective in this respect as the nucleosides.

The effects described in this paper are all produced in a comparatively short time, namely in one hour or less. It is therefore unlikely that these substances exert their effect through being incorporated into the nucleic acids of the cell, and thereby altering their composition and behaviour. Some

information about the effects of the purines on tissue cultures over a longer period has been obtained by Hopkins and Simon-Reuss (7), who found that hypoxanthine increased, and adenine decreased the rate of cell division when these compounds were added to the medium of cultures at a concentration of 50 γ per ml, which is considerably less than those used in the course of this work.

While these experiments were in progress, Professor Green and Dr. Stoner (6) published their monograph of 'The biological actions of the adenine nucleotides,' which admirably describes the extensive work which they and others have done on the pharmacological effects of these substances. The first observations in this field were made by Drury and Szent-Györgyi (4), who found that an extract of cardiac muscle has a strong depressant action on the beating heart and showed that the effective substance was muscle adenylic acid (ASMP). One of the early observations of Green and Stoner (6) was that an extract of skeletal muscle, when injected intravenously, induced a shock-like condition and that here the operative substance was adenosine triphosphate (ATP). It is not necessary, nor would it here be possible to summarise their investigations, but a comparison will be made between some of the effects of nucleosides and nucleotides on the single cell and on tissues and organs.

It must be borne in mind that the concentrations used by Green and Stoner are much smaller than those which I have found necessary. Thus a pronounced bradycardia results when the isolated rabbit heart is perfused with 1 : 500 000 adenosine—which is about one hundredth the minimum dose needed to break up nucleoli in a chick tissue culture. The rate of beating of the embryonic chick heart is also reduced or inhibited by adenosine. Fragments of the 9-day embryonic heart, which continued to beat when cultivated, were affected only by concentrations of adenosine sufficient to break up the nucleoli of the cells of the outgrowth.

In general, the compounds which exert the effects described in this paper are purines and their derivatives, or purine analogues with amino- or amino-groups in the molecule. Amino acids, such as glycine and histidine do not have any of these actions, nor does the guanine analogue guanazolo (8-azaguanine) which resembles guanine except that the imidazole ring is replaced by a ring of three nitrogen atoms. This substance is without effect on embryonic cells in culture under the conditions of the present experiments though it has been used as a competitive antagonist for guanine in studies on growth inhibition (13). Skipper *et al.* (15) find that purine synthesis in the whole mouse is blocked by 8-azaguanine.

The addition of sugars other than *d*-ribofuranose to the molecule of adenine results in inactive compounds. Both the xyloside and the glucoside are without action on tissue cultures under the conditions of these experiments; Green and Stoner (6) also found that the latter was inert. The most interesting derivative to test would have been the ribopyranoside of adenine but this was not available. Alike in the pentose nucleic acids, in the adenylic acids, and in the coenzymes, *d*-ribose is present in the furanose form. Astbury (2) has given stereochemical reasons why this should be so in the nucleic acids.

Adenine has little or no pharmacological effect, but a definite action on dividing cells in tissue cultures, which is not shown by adenine-glucoside. Benzimidazole shows both an adenine-like, and an adenosine-like effect, both of which are lost when this compound is joined to glucose. The one pharmacological action of benzimidazole at present known is that it induces anaesthesia (17). The addition of *d*-ribose to adenine in equimolecular proportion did not modify the effect of the latter substance on cells in tissue cultures.

In comparing the actions of the various nucleosides and nucleotides on cells, we find as did Green and Stoner in their researches, that the derivatives of adenine are more effective than those of other purines. Adenosine is more than ten times as effective as guanosine in breaking up nucleoli, while xanthosine does not exhibit this action at all. No nucleoside or nucleotide other than those of adenine appear completely to inhibit the entry of cells into mitosis under the conditions of these experiments.

We must now compare the relative effectiveness of adenosine and the adenine nucleotides in their various actions on cells, tissues and organs. As pre-prophase inhibitors, adenosine, yeast and muscle adenylic acids, and ATP are, mol for mol, equally effective. This is true also at higher levels of organisation for some effects on the heart and blood vessels, such as that on the heart rate in the rat and on coronary flow in the rabbit (6). The nucleoli in tissue cultures, however, are much more readily affected by adenosine than by the nucleotides. Here, permeability of the cell membrane is probably an important factor. Adenosine, being unionised, would presumably enter the cell more rapidly than the ionising nucleotides, and to this its greater effect on nucleoli may possibly be ascribed. Should this be so, then it could follow that the site of inhibition of cells from entry into mitosis is at the cell membrane.

Nucleotide shock, on the other hand, is much more readily induced by ATP than by the lower nucleotides, or by adenosine. Green and Stoner (6)

have shown that the presence of phosphate is concerned with the shock-inducing action of ATP, for this effect may be reproduced by sodium pyrophosphate alone. Bullough and Green (3) state that both in ischaemic and ATP-induced shock in the mouse the cells of the ear epidermis are temporarily prevented from entering mitosis. It is thus of some interest that nucleotides can inhibit mitosis in both tissue cultures and in the whole animal. Further investigation is necessary to compare the mode of action of these substances in each case, and to see whether any differences which emerge are correlated with the two types of cellular environment.

One distinction between the action of adenosine and the nucleotides on the whole animal and on cultures is in relation to the influence of magnesium, which greatly augments most of the former effects (6). I was unable to demonstrate any comparable action of extra magnesium in tissue cultures, a series of which grew normally in a medium to which was added 10–20 times the normal concentration of this ion. These cultures reacted to given concentrations of adenosine in a similar manner to those grown in a coagulum of normal composition.

With the two types of inhibitory action of adenosine and its nucleotides on the heart and vessels and on the cell, may be bracketed a third, namely the inhibition of preparations of succinic dehydrogenase, described by Adler, Euler and Skarzynski (1) and by Zittle (18). Here, ribonucleic acid also is effective and desoxyribonucleic acid is still more so.

Hence we say that the derivatives of adenine show activity (mainly of an inhibitory kind) at several levels of biological organisation, and that these are indications that the mode of action at each level is not identical in each case. Further research is needed on each type of action, and results from one may help to illuminate the other.

SUMMARY

1. The effect of the addition of chemical substances to living cells in chick tissue cultures was studied by phase-contrast cinematography.

2. The substances used were selected from the purines and their analogues; pyrimidines, nucleosides, and the adenylic acids. The concentrations used ranged from 0.4–9.0 m Mols.

3. Adenosine and the adenylic acids inhibit cells from entry into prophase. The nuclei of intermitotic cells fragment in cultures treated with these substances, or with guanosine or benzimidazole.

4. Adenine, 2,6-diaminopurine, and benzimidazole prevent cleavage and partially inhibit anaphase movement, while reconstruction of the chromosomes proceeds.

I am greatly indebted to Professor H. N. Green and to Dr Stoner of Sheffield, and to my colleagues, Dr H. B. Fell, Dr M. Webb and Mr J. C. Heath for helpful discussions and much assistance during the course of this work.

REFERENCES

1. Adler, E., Euler, H., and Skarzynski, B., *Arkiv Kemi, Min., Geol., 17 A* (1943).
2. Astbury, W. T., *Symposia Soc. Exptl. Biol.*, 1, **66** (1947).
3. Bullough, W. S., and Green, H. N., *Nature*, **164**, 795 (1949).
4. Drury, A. N., and Szent-Györgyi, A., *J. Physiol.*, **68**, 213 (1929).
5. Fell, H. B., and Hughes, A. F., *Quart. J. Microscop. Soc.*, **90**, 355 (1949).
6. Green, H. N., and Stoner, H. B., Biological actions of the adenine nucleotides. Lewis, London, 1950.
7. Hopkins, F. G., and Simon-Reuss, I., *Proc. Roy. Soc. (London), Ser. B*, **132**, 253 (1944).
8. Hughes, A. F., *Quart. J. Roy. Microscop. Soc.*, **69**, 53 (1949).
9. —— *ibid.*, **69**, 215 (1949).
10. —— *ibid.*, **91**, 251 (1950).
11. Hughes, A. F., and Fell, H. B., *ibid.*, **90**, 37 (1949).
12. Jacobson, W., and Webb, M., *J. Physiol.*, **112**, 2 P (1951).
13. Kidder, G. W., and Dewey, V. C., *J. Biol. Chem.*, **178**, 383 (1949).
14. Rosenfeld, M., *Arch. Exptl. Zellforsch. Gewebezücht.*, **12**, 307 (1932).
15. Skipper, H. E., Mitchell, J. H., Bennett, L. L., Newton, M. A., Simpson, L., and Eidson, M., *Cancer Research*, **11**, 145 (1951).
16. Wilson, E. B., *Arch. Entwicklungsmech. Organ*, **13**, 353 (1902).
17. Woolley, D. W., *J. Biol. Chem.*, **152**, 225 (1944).
18. Zittle, C. A., *ibid.*, **162**, 287 (1946).

STUDIES IN HISTOCHEMISTRY

BO G. MALMSTROM[1] and DAVID GLICK

Department of Physiological Chemistry, The Medical School, University of Minnesota, Minneapolis, Minn.

Received May 21, 1951

XXII. THE QUANTITATIVE HISTOLOGICAL DISTRIBUTION OF ZINC IN DOG GASTRIC MUCOSA[2]

A possible correlation of the zinc content with carbonic anhydrase activity of tissues has been of interest ever since Keilin and Mann (4) showed that the enzyme contains 0.33 per cent zinc. Since zinc exists in tissues in forms other than carbonic anhydrase, such a correlation cannot always be found, as indicated by the studies of Leiner (7) and of Scott and Mendive (12). However, in the case of red blood cells, Vallee *et al.* (13) were able to establish a direct parallelism between the zinc concentration and carbonic anhydrase level. If the cells containing the enzyme can be isolated, or at least concentrated, it may be possible to find a parallelism in other cases as well. For blood cells the separation is simple, but for most tissues a histochemical approach is necessary. Recently, the present authors described a quantitative histochemical method for the determination of zinc (11) which has been applied in this investigation to a study of the zinc distribution in the dog gastric mucosa. Davenport (1) previously correlated the presence of carbonic anhydrase in the cat and rat gastric mucosa with the occurrence of parietal cells; the epithelial cells contained some, but much less of the enzyme. This communication will be concerned with the quantitative distribution of zinc with respect to different types of mucosal cells in the dog stomach.

MATERIAL AND METHODS

The dog stomachs used were obtained from normal animals through the courtesy of Drs. R. W. Toon and F. S. Cross, Department of Surgery, University of Minne-

[1] Data in this paper were presented in a thesis submitted to the Graduate Faculty of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] Aided by grants from the National Cancer Institute, U.S. Public Health Service, and the Research Fund of the Graduate School, University of Minnesota.

sota. Pieces of the fundus and pylorus were removed from the stomachs and pinned on cardboard with the mucosal surface up. If food particles adhered to the surface, these were washed off with zinc-free saline. To avoid contamination by dust etc., the tissue was stored in closed Petri dishes. Within 5 min. after removal from the animal, the mucosa was frozen in a cold room (—20° C) and stored there until analyzed. Once frozen, it was not allowed to thaw out. The following procedure is, in general, patterned after that described by Linderstrøm-Lang, Holter, and Søeborg-Ohlsen (9).

For analysis, a cylinder of the frozen tissue, with its long axis at right angles to the mucosal surface, was punched out with a plastic borer (4 mm inner diameter) (11). The cylinder was frozen with a drop of zinc-free saline to a carbon dioxide cooled freezing head clamped in the jaws of the blockholder of a rotary microtome. The tissue was anchored to the freezing head at the muscle end. Before cutting, the thickness of the mucosa was measured to 0.1 mm. Serial sections 30 μ thick were cut with a glass knife (6). Because of some uneveness of the surface, the fifth section was usually the first complete one. Separate sections taken from different levels in the mucosa were put in individual quartz tubes and carried through the colorimetric dithizone procedure for the determination of zinc previously described (11). The first section after each cooling was never used for analysis because of its indeterminate thickness due to the contraction of the metal of the freezing head. A control experiment was carried out in which 2 sections cut at 15 μ were taken following each 30 μ section. From Fig. 1, it may be seen that whether the points on the zinc distribution curve were taken from analyses on single 30 μ sections or on 2 of 15 μ made no difference. Six cylinders from the fundus and 3 from the pylorus were taken from 4 stomachs for analysis.

A ring of tissue (10 mm outside diameter) was punched out around the hole left after the cylinder was removed for analysis. This tissue was fixed in 10 per cent formalin, dehydrated in graded alcohols, and embedded in paraffin in the usual manner. It was then cut into halves through the center of the hole in a direction perpendicular to the mucosal surface. Ten μ sections were cut parallel to the long axis, deparaffinized, and stained with hematoxylin-eosin. These sections were used to determine the relative depth from the mucosal surface of the region of maximum concentration of the different types of cell. The distance from the surface was measured with a microscope ocular micrometer. In order to correct for the shrinkage that results from the fixation and dehydration the ratio of the thickness of the fresh frozen to the stained mucosa was measured.

Quantitative Definition of Sample Size. X-ray absorption studies by Engström and Glick (2) on the gastric mucosa from a dog given histamine and by Malmstrom and Engström (10) on material from a normal dog showed that the dry weight per unit volume of the chief cells is about double that of the parietal cells. Therefore, the density of the sections should depend on the relative number of the two types of cells, and thus vary with distance from the surface. Accordingly, volume was not used as a reference quantity, and the results have been referred to the "reduced weight," *i.e.* the wet weight minus the weight of an equal volume of water (3. 8). This was determined in the density gradient tube as described by Linderstrøm-Lang and Holter (3, 8). A section for the "reduced weight" determination was usually taken adjacent to each section used for zinc analysis.

RESULTS

The results of only a number of representative experiments are given. Figs. 2 and 3 give the amount of zinc (mμg) per section (0.38 μl) and the "reduced weight" per section as a function of distance from the mucosal surface in the fundus. The curve representing the amount of zinc per unit "reduced weight" is calculated from the other two curves. Fig. 4 illustrates a similar experiment carried out on pyloric material. The results of another experiment on the fundus and pylorus, with no "reduced weight" data, are shown in Fig. 5.

DISCUSSION

The results demonstrate that the chief and muscle cell regions of the gastric mucosa have a low relatively constant zinc concentration, while the epithelial and parietal cell regions have concentrations that are significantly higher. The "reduced weight" in the epithelial cell region was consistently lower than that in all other portions which means that the zinc content per unit weight in this region is considerably higher than in the parietal cell area. The "reduced weight" values for the sections taken under the epithelial cells are essentially constant.

The high zinc concentration in the epithelial cells may be due primarily to an uptake of the element from the stomach contents. There is some carbonic anhydrase in epithelial cells but much less than is found in the parietal cells (1). The elevated zinc level in the parietal cell region is likely related to its high carbonic anhydrase activity (1).

The average zinc concentration in the chief cell region is 3.0 mμg per section, and in the parietal cell 6.0 mμg per section. This corresponds approximately to 8 and 16 μg per g wet tissue in the 2 regions. Koga (5) reported the zinc concentration for the whole dog stomach to be 10.2 μg per g wet weight.

SUMMARY

The quantitative histological distribution of zinc in the normal dog gastric mucosa in both the fundus and pylorus was determined. The highest zinc concentrations per unit "reduced weight" were found in the epithelial and parietal cell regions, the former exceeding the latter. It is possible that the high values in the epithelial cell material resulted largely from uptake from the stomach contents and to a lesser degree from the occurrence of a certain

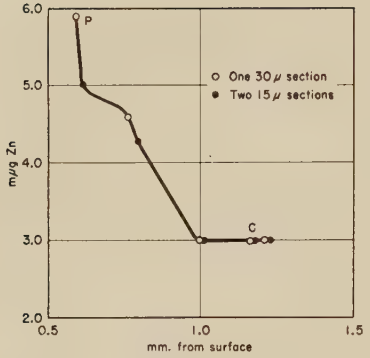


Fig. 1.

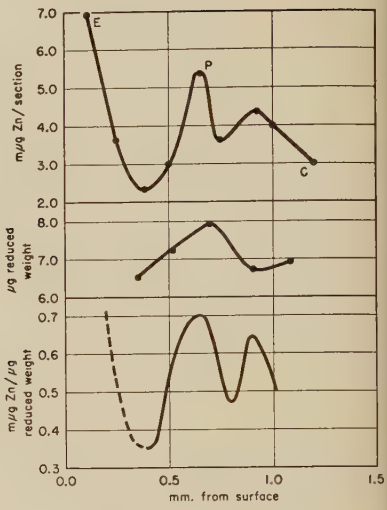


Fig. 2.

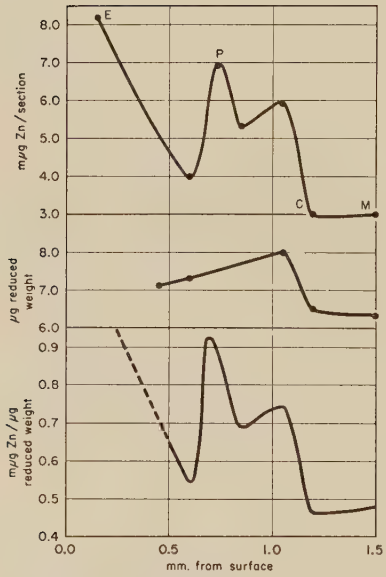


Fig. 3.

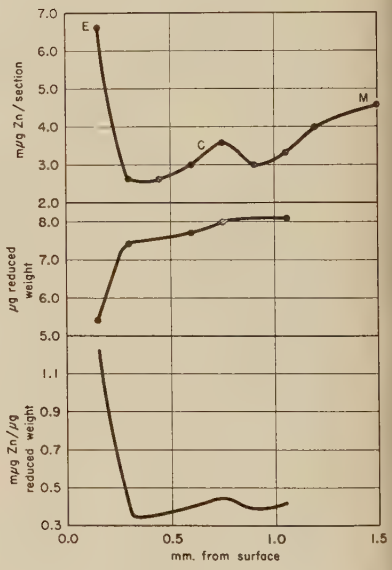


Fig. 4.

Fig. 1. Zinc distribution in fundic mucosa of dog stomach No. 4. In this and following figures regions of maximum concentration of cells indicated by (E) epithelial, (P) parietal, (C) chief, and (M) muscle.
Fig. 2. Zinc distribution in fundic mucosa of dog stomach No. 1.
Fig. 3. Zinc distribution in fundic mucosa of dog stomach No. 2.
Fig. 4. Zinc distribution in pyloric mucosa of dog stomach No. 2.

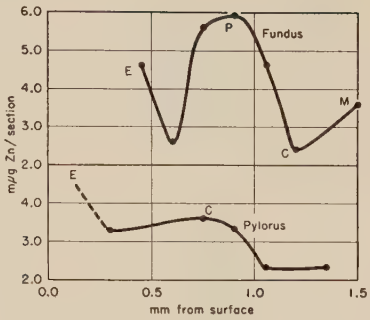


Fig. 5. Zinc distribution in fundic and pyloric mucosa of dog stomach No. 3.

amount of carbonic anhydrase, but the high level in the parietal cell portion may be related primarily to the maximum concentration of carbonic anhydrase which occurs in this region of the fundic mucosa.

REFERENCES

1. Davenport, H. W., *J. Physiol.*, **97**, 32 (1939).
2. Engström, A., and Glick, D., *Science*, **111**, 379 (1950).
3. Glick, D., Techniques of Histo- and Cytochemistry. Interscience Publishers, New York, p. 420, 1949.
4. Keilin, D., and Mann, T., *Biochem. J.*, **34**, 1163 (1940).
5. Koga, A., *Keijo J. Med.*, **5**, 97 (1934).
6. Latta, H., and Hartmann, J. F., *Proc. Soc. Exptl. Biol. Med.*, **74**, 436 (1950).
7. Leiner, M., *Klin. Wochenschr.*, **22**, 130 (1943).
8. Linderstrøm-Lang, K., and Holter, H. in Bamann, E., and Myrbäck, K., Die Methoden der Fermentforschung, Thieme, Leipzig, pp. 1132–1162, 1940.
9. Linderstrøm-Lang, K., Holter, H., and Søeborg-Ohlsen, A., *Compt. rend. lab. Carlsberg, Sér. chim.*, **20**, 66 (1935).
10. Malmstrom, B. G., and Engström, A., unpublished data.
11. Malmstrom, B. G., and Glick, D., *Anal. Chem.* **23**, 1699 (1951).
12. Scott, D. A., and Mendive, J. R., *J. Biol. Chem.*, **140**, 445 (1941).
13. Vallee, B. L., Aewis, H. D., Altschule, M. D., and Gibson, II, J. G., *Blood*, **4**, 467 (1949).

XXIII. SIMPLE AND EFFICIENT FREEZING-DRYING APPARATUS FOR THE PREPARATION OF EMBEDDED TISSUE[1]

DAVID GLICK and BO G. MALMSTROM

The manifold advantages of the freezing-drying treatment of tissue in preparation for embedding and sectioning in histological, and especially in

[1] Aided by grants from the Division of Research Grants and Fellowships, National Institutes of Health, U. S. Public Health Service, Bethesda, Maryland, and The Medical Research Fund of the Graduate School, University of Minnesota, Minneapolis.

histochemical techniques, have been reviewed earlier (2). Among the designs published in recent years are those of Packer and Scott (5), Taylor (8), Gersh (1), and Wang and Grossman (9). The chief disadvantages that characterize earlier equipment have been the relative complexity of the apparatus, or high cost, or excessive time required to complete the freezing-drying process, or combinations of these. The present study was undertaken to minimize these difficulties and thereby to make more practical an increased application of the freezing-drying technique.

A discussion of the theory of dehydration from the frozen state has been given by Malmstrom (4), and two models of freezing-drying apparatus were developed which incorporate features of design that the theory requires for efficiency. In these models the vacuum system was attached to simple, replaceable, glass freezing-drying units, and a considerable reduction in the time required for processing the tissue was achieved.

During the course of this work, Stowell (7) described a freezing-drying apparatus which embodied the same basic principles employed by the present authors, but with differences in design which will be apparent.

EXPERIMENTAL

I. *Apparatus*

A. *Tissue Holders.* Tissue holders of different designs were devised, and those illustrated in Fig. 1 were the ones finally employed. These are made of stainless steel and are arranged so that separate tissue samples can be placed in the individual rings. Cheese cloth or gauze is fastened under each ring unit to provide a permeable floor on which the samples rest. The cloth is held in place by thread tied around the circumference of the unit, and a groove in the peripheral edge into which the thread can fit facilitates the tying. These grooves may be seen in the side views (A) and (C^1) in Fig. 1. The holder (A) which has removable units (B) has cord wrapped around the ring on the top of the vertical stem to serve as insulation so that it can be picked up with the fingers after the units (B) have been submerged in isopentane cooled by liquid nitrogen. Holders (C) and (D) are lowered into, and raised out of, the isopentane by means of a wire with a hook that fits into the small hole in the top of the vertical stem shown in (C^1). The wooden dowel (E) is used at the end of the process to punch out the paraffin discs, which contain the embedded tissue, from the rings in the tissue holders.

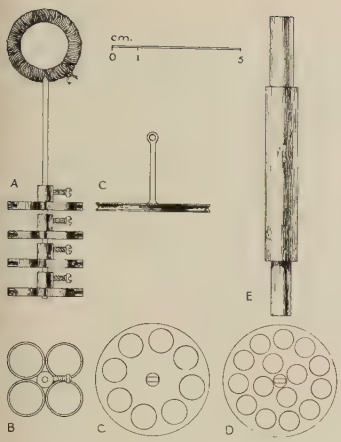


Fig. 1.

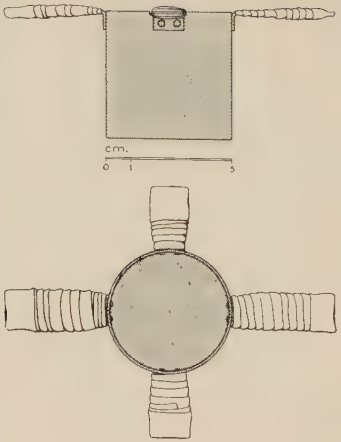


Fig. 2.

Fig. 1. Tissue holders (A, B, C, D) and Dowel (E) for punching out paraffin discs.
Fig. 2. Isopentane container.

B. *Isopentane Container.* The stainless steel container that holds the isopentane for the initial freezing of the tissue is shown in Fig. 2. The 4 arms at the top of the vessel are wrapped with adhesive tape to enable handling even when the metal is at liquid nitrogen temperature. By means of these arms, which are made to rest on the mouth of a Dewar flask containing liquid nitrogen, the vessel can be surrounded with the cooling liquid. The tissue holders fit into the vessel and the rings holding the samples are submerged in the cold isopentane. The Dewar flask employed had an inside diameter of 6.8 cm and a height of 15 cm.

C. *Freezing-Drying Unit No. 1.* Two Pyrex glass freezing-drying units were designed, the first of which is illustrated in Fig. 3. The removable sample tube (A) has a standard taper ground glass joint to enable the use of several different tubes. The trap (C) has a short inner tube (D) to reduce the distance the water molecules have to travel before being trapped on the sides of tube (C) which is surrounded by liquid nitrogen contained in a Dewar flask. The limiting factor in the proximity of (A) and (C) is the fact that each tube must be surrounded by liquid in a Dewar flask, and hence sufficient space must be allowed between the tubes to permit the two Dewar flasks to stand side by side. The Dewar flask used to surround tube (A) had an inside diameter of 6.8 cm and a height of 30 cm, while the one used to surround trap (C) had corresponding dimensions of 11×30 cm.

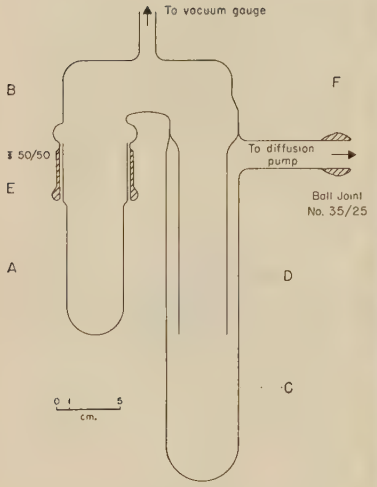


Fig. 3.

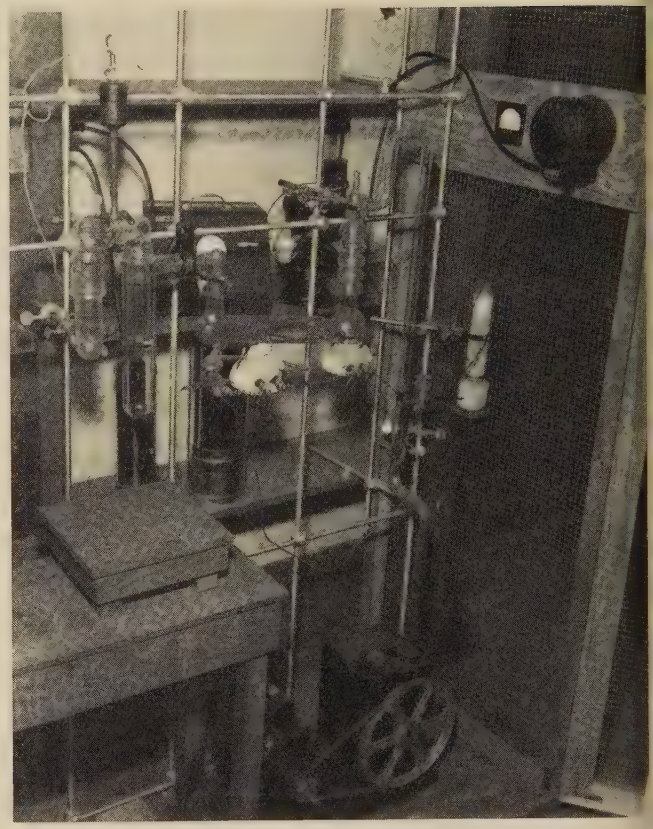


Fig. 4.

Fig. 3. Glass freezing-drying unit No. 1.
Fig. 4. Complete assembly of freezing-drying unit No. 1.

The former is employed for a solid carbon dioxide-organic liquid cooling bath. The liquid nitrogen evaporates rather rapidly and this necessitates a much larger Dewar flask in order that sufficient of the liquid be retained overnight.

The standard ball joint (F) joins the apparatus to the diffusion pump which was a glass, 2 stage, air-cooled pump having a capacity of 20 L/sec. at 10^{-4} mm Hg using "Octoil-S" as the pump fluid (No. GF-20 A, Vacuum Equipment Division, Distillation Products, Inc., Rochester, New York). A standard glass socket joint to fit the ball joint (F) was sealed to the high vacuum end of the diffusion pump since the latter does not have this feature when purchased. A pinch clamp (Size 35, No. 3241 Arthur H. Thomas Co., Philadelphia, Pa.) was used to hold the ball and socket joint together. The 110 volt A.C. current supplied to the pump heaters was controlled by a

variable transformer with a 750 watt capacity, and an A.C. ammeter with a range of 0–5 amps. was placed in series with the heaters.

The mechanical fore-pump used with the diffusion pump was a 2 stage, electric motor-driven pump with a free air capacity of 33.4 L/min. (Duo-Seal, No. 1405 H, W. M. Welch Scientific Company, Chicago, Ill.). A glass high vacuum stopcock was sealed into the glass tube connecting the diffusion pump to the fore-pump so that the vacuum in the system could be broken at that point. The open end of the stopcock was connected to a column of anhydrous calcium sulfate (Drierite, W. A Hammond Drierite Company, Xenia, Ohio) to remove moisture from the air admitted to the apparatus. This stopcock was lubricated by silicone high vacuum grease (Dow Corning Corporation, Midland, Mich.). The ground glass joints (E, F) in the apparatus were also lubricated with this grease which proved preferable to the other types of vacuum greases tested.

A Philips glow-discharge type of ionization gauge was employed to measure the vacuum (Type PHG-1, Distillation Products, Inc.). The metal tube of the instrument was supplied with a metal to glass seal so that it could be attached to the glass apparatus. This type of gauge has the advantage that the tube will not burn out if the vacuum is suddenly broken while the tube is in use. On the other hand, it can register pressures down to only 1×10^{-5} mm Hg and lower pressures are actually attained in the apparatus. However, it is sufficient to know that the pressure is less than 1×10^{-5} mm Hg without knowing how much less. Ionization gauges that indicate lower pressures are the type that burn out when the vacuum is broken while the tube is in use.

A photograph of the complete equipment is given in Fig. 4. "Glyptal," red enamel paint (No. 1201 General Electric Co., Schenectady, N. Y.) was coated over the glass-metal seal to the vacuum gauge, and also over the rubber pressure tubing used to connect the fore-pump to one end of the glass tube sealed to the diffusion pump.

D. *Freezing-Drying Unit No. 2.* This freezing-drying apparatus was subsequently designed to further satisfy theoretical requirements for maximum efficiency. The glass unit, illustrated in Fig. 5, differs principally from that shown in Fig. 3 in the use of a liquid nitrogen water trap (D) which is positioned only a few centimeters from the sample. The shorter distance the water molecules have to travel from sample to trap without the presence of angles between them, makes for considerably faster dehydration. In this design the liquid nitrogen has to be added to the trap every few hours to keep the chamber (D) filled. However, only a few hours are required for

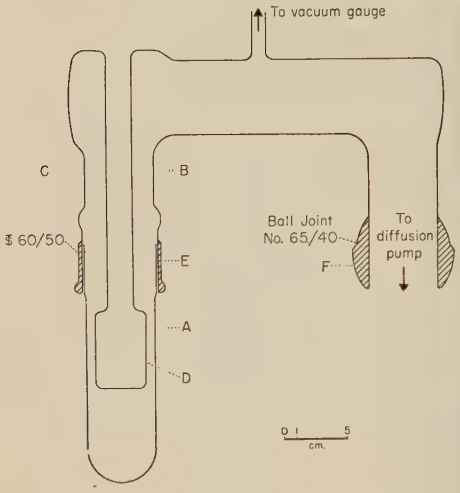


Fig. 5.

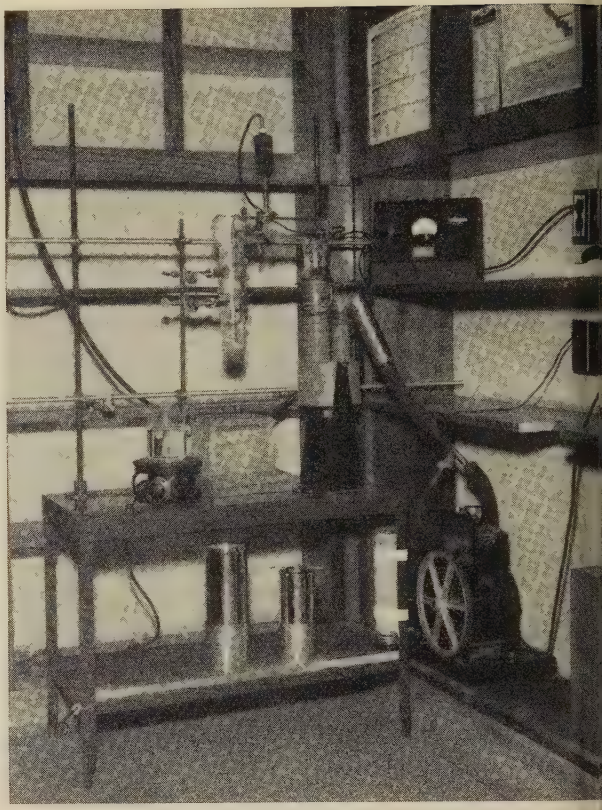


Fig. 6.

Fig. 5. Glass freezing-drying No. 2.
Fig. 6. Complete assembly of freezing-drying unit No. 2.

the operation so this does not become a great inconvenience. The Dewar flask containing solid carbon dioxide and an organic liquid, which is used to cool tube (A) has an inner diameter of 8.5 cm and a height of 20 cm. Different organic liquids were employed to obtain temperatures from —15° to —80°.

A Philips vacuum gauge is also used with this apparatus, and the oil diffusion pump is a water-cooled all metal pump with a capacity of 50 L/sec. at 1×10^{-4} mm Hg using "Octoil-S" pump fluid (No. VMF-50W, Distillation Products, Inc.). Since this pump is designed to give maximum performance when the heater is operated at 110 volts A.C., no current controlling instrument or meter was used with it. As in the other apparatus, Fig. 4, a Welch fore-pump was employed, as well as a drying column to remove moisture from the air admitted to the system when the vacuum is broken by opening

the stopcock interposed between the fore- and diffusion pumps. A standard socket joint of brass was made to fit the ball joint (F), and the brass joint was silver-soldered to the diffusion pump. A brass adapter was also silver-soldered to the low vacuum end of this pump to connect it with the glass stopcock as shown in Fig. 6, and a short heavy piece of pressure tubing was used to join the fore-pump to the other arm of the stopcock.

"Glyptal" was coated over the rubber connection, the glass-metal seal to the vacuum gauge, and the ball and socket joint between the glass freezing-drying unit and the diffusion pump. The high vacuum silicone grease was used on the ground surfaces of joint (E) and "Plicene" cement (Central Scientific Co., Chicago, Ill.) was used to seal joint (F), Fig. 5.

II. *Procedure*

A. *Degassing the Paraffin Embedding Medium.* The sample tube is prepared in advance for the dehydration-embedding procedure by placing paraffin embedding medium ("Tissuemat," m.p. 50–52°, Fisher Scientific Co., Pittsburgh, Pa., was used) in the tube to a depth of about 4 cm, attaching to the apparatus, turning on only the fore-pump, and then melting the paraffin by surrounding the tube with a beaker of hot water. As the paraffin melts, gas bubbles will be evolved, and the degassing is continued until the bubbling of the completely melted paraffin ceases. With the fore-pump still running, rapid solidification is effected by surrounding the tube with the Dewar flask containing the solid carbon dioxide-organic liquid mixture that is used later for cooling during the dehydration. Only when the paraffin is entirely solid is the fore-pump turned off and the vacuum broken.

B. *Initial Freezing of Tissue.* Samples of tissue immediately after removal from the organism are cut into small pieces which should not exceed 2 mm in thickness, placed on the gauze bottom of a tissue holder with each piece in a separate ring, and the holder with its samples is submerged in the isopentane previously cooled with liquid nitrogen. Isopentane will eventually freeze to a solid mass at the temperature of liquid nitrogen but the tissue is frozen so quickly that this part of the process can be finished before the isopentane becomes solid. If delays are encountered, the isopentane can be kept in the state of a half-frozen mush by removal from the liquid nitrogen bath until signs of solidification disappear. It is satisfactory to use the half-frozen mush for the tissue freezing. Liquid air, which has a higher temperature than liquid nitrogen and which would be less apt to freeze the isopentane, was not employed because of the explosion hazard attending its use.

After about 30 sec. in the freezing liquid, the tissue holder with its samples

is quickly transferred to the freezing-drying apparatus and set on a block of previously degassed paraffin in the sample tube.

C. *Dehydrating the Frozen Tissue.* After the tissue has been frozen in the isopentane, the sample tube is removed, the tissue holder is quickly placed on the paraffin block, and the tube is immediately connected again to the apparatus. The Dewar flask with the solid carbon dioxide and organic liquid mixture is raised to surround the tube at once to prevent the tissue from thawing. The Dewar flask containing liquid nitrogen is raised to surround the water trap in Unit No. 1, or the liquid nitrogen is poured into the water trap in Unit No. 2. The fore-pump is then started, and subsequently the diffusion pump is turned on. The time required for the dehydration will vary with the amount, dimensions, and kind of tissue, and hence the time must be determined empirically in each case. In the experience of the authors, complete dehydration of 4 pieces of dog gastric mucosa (about 2 mm cubes) was observed after 6 hrs. when the dehydration was carried out at —80° and 1×10^{-5} mm Hg. in Unit No. 2; about 2 days were necessary with Unit No. 1 under these conditions. At —40° and 1×10^{-5} mm Hg., in Unit No. 2 complete dehydration was obtained after 2 hrs., but 12 hrs. was insufficient at —80° and 1×10^{-3} mm Hg, thus the advantage of employing a pressure of 1×10^{-5} rather than 1×10^{-3} mm Hg. at —80° is apparent, and this is in accord with the theory presented by Malmstrom (4). Tissues with higher lipid content required longer times, *e.g.*, 8 hrs. at —22° and 1×10^{-5} mm Hg. was needed for comparable size pieces of rat adrenal.

D. *Embedding the Dehydrated Tissue.* After the tissue has been dehydrated, the Dewar flask surrounding the sample tube is removed and the tube with its contents is allowed to warm up at room temperature for about five minutes. Then the tube is surrounded with a beaker of water warmed on a thermostatically controlled electric hot plate to melt the paraffin. To avoid overheating the paraffin and damaging the tissue, the heating is controlled so that some solid paraffin remains at the bottom of the tube. As the paraffin melts the tissue holder will sink under the liquid and, *if the hydration has been complete, no gas bubbles will arise from the tissue.* Since this part of the process is carried out with the vacuum pumps still running, gas bubbles will be evolved as long as moisture remains in the samples. If the bubbling continues for more than a few minutes, the material has been insufficiently dehydrated for reliable histochemical use and should be discarded.

After the tissue has been entirely submerged in the molten paraffin, in-

filtration is allowed to proceed for about five minutes. Then the diffusion pump is turned off, and after it has cooled, the fore-pump is also turned off and air is admitted to the system through the drying column. The beaker of hot water is removed to allow the paraffin to solidify. Finally, the sample tube is disconnected from the apparatus and warmed with hot water to separate the paraffin block from the glass wall so that the block can be removed. The excess paraffin is trimmed away, and the wooden dowel is used to push out the separate paraffin discs from the tissue holder rings.

III. *Freezing temperatures during dehydration*

Solid carbon dioxide-acetone mixtures were first used to maintain the tissue in the frozen state during the dehydration. The low temperature (—80°) of these mixtures necessitated a prolonged dehydration time. The time decreases rapidly as the temperature rises above —40° (4). However, if the temperature employed is too high morphological and chemical alterations may occur. Unfortunately, no particular temperature can be set as the highest that can be used with safety. Certainly temperatures below —60° would be completely safe since the lowest temperature that might be expected in a eutectic mixture of tissue salts would probably be not lower than this value. However, in order to shorten the dehydration time, the freezing mixtures listed in Table I may be used to cool the samples. Furfuryl alcohol[1] and the following compounds[2], propylene glycol o-chlorophenyl ether, propylene glycol p-sec-butylphenyl ether, propylene glycol o-sec-butylphenyl ether, ethylene glycol phenyl ether acetate and ethylene glycol p-tert-butylphenyl ether acetate were also tried with solid carbon dioxide, but none proved suitable because they became highly viscous liquids (furfuryl alcohol became solid) whose temperatures were not constant in the presence of excess solid carbon dioxide. Ethyl cyclohexane[2] behaved like acetone, giving a temperature of —80°, and it had the advantage of low volality (130–134°). Of course, mechanical refrigeration could be employed in place of the solid carbon dioxide-organic liquid mixtures, but this would involve considerable additional expense.

It would remain to be established in the case of any given tissue whether morphological or chemical change had occurred during dehydration at freezing temperatures above —60°. While, theoretically, it might be argued that temperatures of the order of —60° or lower should be used, in practice

[1] Kindly furnished through the courtesy of Quaker Oats Co., Chicago.

[2] Kindly furnished through the courtesy of Mr. A. W. Smith, The Dow Chemical Co., Midland, Michigan.

TABLE I

No.	Compound	Temperature of Compound-Solid CO_2 Mixture Before Complete Freezing
1.	Ethylene Glycol	-15°
2.[1]	Dibutoxytetraglycol	-22°
3.	Dimethoxytetraglycol	-31°
4.[2]	Diethylene Glycol	-37°
5.	Ethylene Dichloride	-40°
6.	Ethylene Diacetate	$<-50^\circ$

No. 2 is, but No. 3 is no longer, available from Carbide and Carbon Division, Union Carbide and Carbon Corp., New York. Toxicity of the vapors of No. 5 should be considered in its use. See Wikoff *et al.* (10) for additional information concerning organic liquids to be used with solid carbon dioxide.

it might be possible to employ temperatures of the order of —20° to —40° without demonstrable harm. Simpson (6) reported that somewhat better morphological results were obtained at —40° than at —30°, while little difference could be observed between —40° and —50°. Hoerr and Scott (3) favored temperature below —30° and Gersh (1) stated that temperatures of —30° to —40° are usually suitable for most problems. Stowell (7) used —30° or colder. All would probably agree that for highly diffusible ions temperatures as low as —60° would be the safest.

DISCUSSION AND SUMMARY

The need for frequent replacement of liquid nitrogen in the water trap in Unit No. 2, would become a problem for samples that required a dehydration time longer than 8–10 hrs. This difficulty might be circumvented by inserting a trap such as (C), Fig. 3, between (B), Fig. 5, and the ball joint which connects to the diffusion pump. The resulting apparatus would be a combination of Units No. 1 and 2. Slowly drying samples could be first dehydrated overnight with no liquid nitrogen in the trap (D), Fig. 5, but with liquid nitrogen in the Dewar flask surrounding the inserted trap. Then, in the morning, liquid nitrogen could be added to trap (D), Fig. 5, and the dehydration finished with both traps cooled.

The freezing-drying units described, which were designed to meet the theoretical requirements for efficiency, have been found to minimize the cost and complexity of the apparatus, and the time required for the dehydra-

[1] Kindly furnished through the courtesy of Mr. B. B. Lupton, Carbide and Carbon Chemicals Corp., Chicago.

[2] Kindly furnished through the courtesy of Mr. V. I. Cooper, The Dow Chemical Co., Chicago.

tion process. This was accomplished by: 1. use of vacuum pumps with greater capacity than seems necessary in order to render slight leaks unimportant and to shorten the time required to attain high vacuum, 2. a reduced distance between tissue sample and water vapor trap to hasten rate of dehydration, 3. a reduced water vapor tension in the trap to speed dehydration and make it more complete, and 4. apparatus of relatively large bore with a minimum of joints and angles to speed evacuation.

REFERENCES

1. Gersh, I., *Bull. Intern. Ass. Med. Museums*, **28**, 179 (1948).
2. Glick, D., Techniques of Histo- and Cytochemistry, Interscience Publishers Inc., New York, pp. 3–5, 1949.
3. Hoerr, N. L., and Scott, G. H., in Medical Physics, O. Glasser editor, Year Book Publishers, I, p. 466, 1944, II, p. 292, 1950.
4. Malmstrom, B., *Exptl. Cell Res.*, **2**, 688 (1951).
5. Packer, D. M., and Scott, G. H., *Bull. Intern. Ass. Med. Museums*, **22**, 85 (1942).
6. Simpson, W. L., *Anat. Rec.*, **80**, 173 (1941).
7. Stowell, R. E., *J. Natl. Cancer Inst.*, **10**, 1361 (1950).
8. Taylor, A. C., *J. Lab. Clin. Med.*, **29**, 657 (1944).
9. Wang, K. J., and Grossman, M. I., *ibid.*, **34**, 293 (1949).
10. Wikoff, H. L., Cohen, B. R., and Grossman, M. I., *Ind. Eng. Chem., Anal. Ed.*, **12**, 92 (1940).

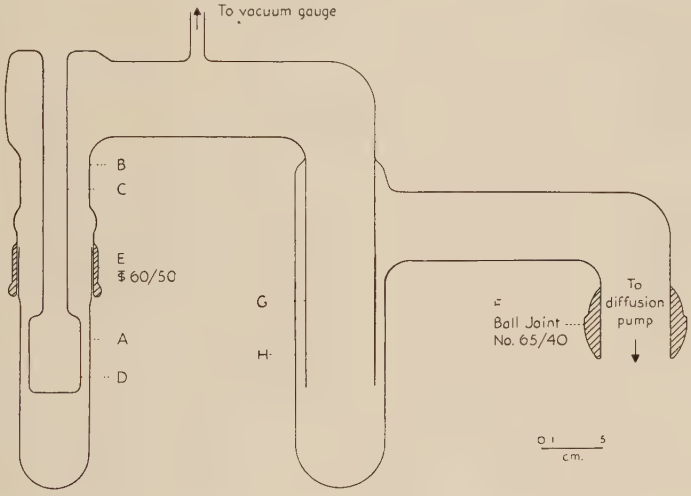


Fig. 7.

ADDENDUM

After the original manuscript was submitted for publication, the apparatus illustrated in Fig. 7 was constructed in line with the suggestion made in the first paragraph of the discussion. This design has proved very useful for tissues with a high fat content.

THE ORIGIN OF CERTAIN NUTRITIVE SUBSTANCES IN THE EGGS OF HEMIPTERA

FRANZ SCHRADER and CECILIE LEUCHTENBERGER

Department of Zoology, Columbia University, New York, and Institute of Pathology, Western Reserve University, Cleveland, Ohio

Received June 10, 1951

The metabolism of early developmental stages has long been of interest to biologists. In order to construct a reliable foundation for studies of this sort several workers (for instance Alfert (1), Lison and Pasteels (10)) have endeavored to determine the quantitative relationship of such important constituents as desoxyribosenucleic acid (DNA), ribosenucleic acid (RNA) and proteins in the rapidly multiplying cells of the young embryo. The methods that are involved in such determinations are photometric in character and are based on the discovery of Caspersson (2) who showed that the microscope could be adapted for the chemical analysis of intracellular substances *in situ*.

Such an approach to the general problem has also been utilized in the present investigation, which is however more specifically concerned with the ovarial conditions through which the egg is supplied with the substances that are later used by the developing embryo. These nutritive materials which are contained in the egg at the time of fertilization are generally covered by the term "yolk" though it is patent that several different constituents are involved. The complexity in the nutrition of the embryo is already apparent from such cases where whole cells are taken into the ovum (as for instance in certain flat worms, crustaceans, and insects). The ready utilization of these "nurse cells"—nucleus as well as cytoplasm— by the embryo indicates clearly enough that the lipoidal components which often constitute the main bulk of the egg, are not the only ones which may serve in embryonic nutrition.

In the majority of species, these nutritive materials are provided by the follicle cells that surround the growing egg in the ovary. Since, as already stated, we are dealing with a complexity of substances, this activity of the follicle cells is in itself no simple process. For that reason it is fortunate that in some species the food materials in the egg are derived from two different

and independent sources, for in this dual origin lies the possibility that different cells contribute different materials. This would simplify our attack on the problem of the nature and origin of nutritive substances since the various processes are certainly more easily analyzed if they do not all occur in a single type of cell.

Conditions such as these are encountered in the ovaries of many Hemiptera and they are especially well represented in the family Coreidae. In these the follicle cells undoubtedly contribute much, perhaps the greater portion, of the nutritive material, but an additional quantity originates in the so-called "end chamber" of the ovary. The gonadal structure that is correlated with these special developments has long been known in its histological aspects and is recognized by entomologists under the term "telotrophic" ovary (17). Our own work, which was begun several years ago, is more specifically concerned with the cytological and cytochemical processes that take place in the end chamber.

MATERIAL AND METHODS

The coreid species most favorable for our studies was *Acanthocephala bicoloripes*, which was collected in Costa Rica during the years 1944 and 1950. Unless specially noted, it is this species to which we shall have reference in the following pages. Judging from the slides in the collection at Columbia University it is likely that the northern species, *Acanthocephala terminalis*, offers similar conditions. Material of certain other coreids such as *Spartocera gigantea* and *Anasa tristis* was used for comparison in connection with certain of the questions that arose.

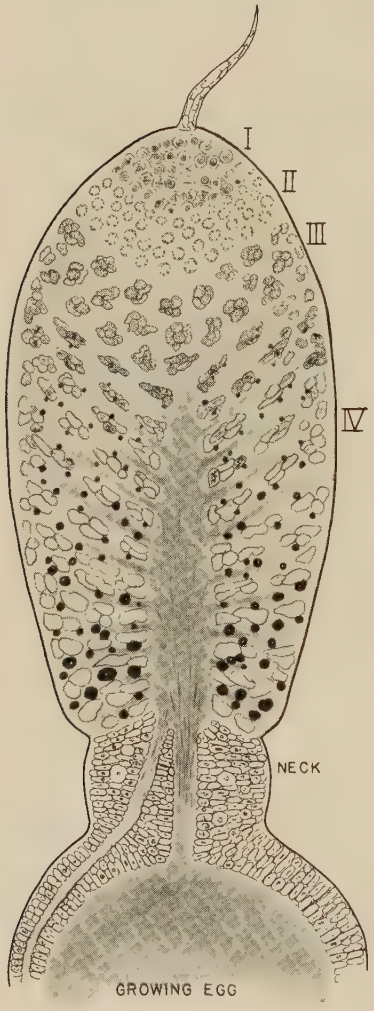
The cytological study was linked with certain cytochemical methods. These comprised the Feulgen reaction and methyl green staining, staining of the cytoplasm and nucleolus with fast green, and use of the polyaldehyde Schiff reaction (PAS) to indicate the presence of polysaccharides. The relative quantities of various substances were determined photometrically (15, 11). For the characterization of the polysaccharides, the PAS reaction was used in conjunction with enzymes as described by Leuchtenberger and Schrader (6).

RESULTS

A. *Zones I and II.* The general topography of the coreid ovary parallels, at least in its general features, the conditions that are encountered in the ovaries of other Hemiptera. The ovary consists of several lobes and each lobe represents a string of growing eggs, increasing in size from the anterior to the posterior end. At the anterior extremity this string is the "end chamber" in which, as already stated, are formed certain of the materials that are

contributed to the growing eggs. The rest of the food materials are derived from the follicle cells that surround each egg. The main features, as just outlined here, were quite adequately described by Gross (3).

The end chamber, with which we are especially concerned, may be arbitrarily divided into four zones (Fig. 1). In Zone I, occupying the anterior region of the chamber, there is much mitotic activity (unless the female has already passed the egg-forming period) and nuclei in all phases are here encountered. The mitotic cycle shows no unusual features and a normal, diploid complement of 22 chromosomes appears in the metaphase plates. During the interphase and prophase there is a nucleolus of the usual size and it has a random placement without special reference to the nuclear membrane. A cell wall or membrane, though thin and delicate in appearance, is nevertheless always present in these cells (Fig. 2). There is some question whether the definitive ova are derived from this zone or from the more posterior "neck" region, which separates the end chamber from the youngest growing egg. The cells of this neck region are likewise undergoing normal mitosis and from our study it would appear that it is they who furnish the ovaria that become the true eggs.



In Zone II, posterior to the one just mentioned, several of these cytological features have undergone a change (Fig. 3). Adnate to Zone I, the nuclei are still of approximately the same size, but further back in the zone there is an increase of the nuclear diameter. In some cases a doubling of the nuclear volume is approached, though the gradation in size is not uniform. What especially gives this region a distinct character is the fact that all of its nuclei seem to have come to rest in a late prophase stage. Without exception these

Fig. 1. Semidiagram of the end chamber and neck region of an ovarial lobe.

Fig. 2. The normal cells in Zone I.
Fig. 3. Nuclei with peripherally placed chromosomal bodies and nucleoli in Zone II. Cell walls have disappeared.
Fig. 4. Gathering of amoeboid nuclei in Zone III. The variable size suggests fusion processes, as does the increase in number of nucleoli.
Fig. 5. Running-together of DNA in the nuclei of Zone IV.
Fig. 6. Extrusion of DNA droplets and nucleoli through the nuclear membrane.
Fig. 7. Nuclei lower in Zone IV, showing increase in size.
Fig. 8. Seepage of DNA through the nuclear membrane of a nucleus in the posterior region of Zone IV.

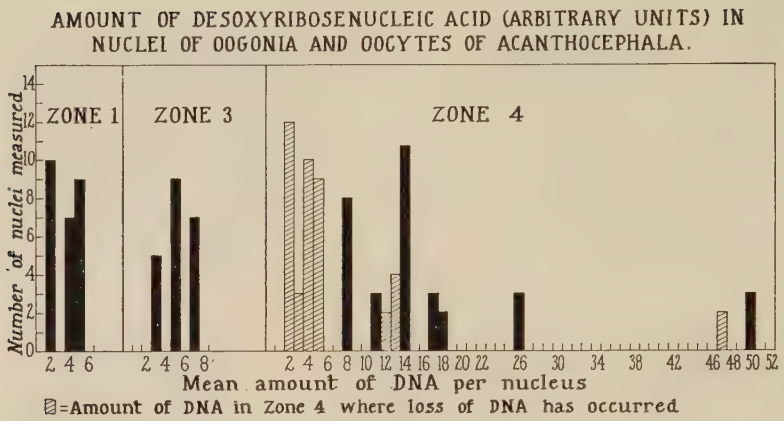

AMOUNT OF DESOXYRIBOSENUCLEIC ACID (ARBITRARY UNITS) IN NUCLEI OF OOGONIA AND OOCYTES OF ACANTHOCEPHALA.
ZONE 1
ZONE 3
ZONE 4
Number of nuclei measured
0 2 4 6 8 10 12 14
2 4 6
2 4 6 8
2 4 6 8 10 12 14 16 18 20 22 26 30 34 38 42 46 48 50 52
Mean amount of DNA per nucleus
=Amount of DNA in Zone 4 where loss of DNA has occurred

Fig. 9. Histogram, to show the amounts of DNA in different zones of the end chamber.

nuclei carry peripherally placed, Feulgen-positive bodies which in number correspond closely to the diploid complement of chromosomes. Almost certainly these chromatin bodies therefore represent chromosomes not quite fully condensed. The nucleolus in such nuclei is, like the chromatin bodies, always in touch with the nuclear periphery so that the central and major portion of the nuclear space is entirely devoid of any chromosomal or nucleolar structure (Fig. 3). It is not possible to recognize any cell membranes in Zone II. The fact that the nuclei remain evenly spaced and separated from each other would seem to indicate that some cellular integrity is maintained but, so far as the purely cytological evidence goes, all these nuclei lie in a common, lightly staining (with fast green), homogeneous cytoplasm.

B. *Zones III and IV.* Whatever the nature of cellular demarcation in Zone II may be, the developments in Zone III denote that any boundaries, possibly so fragile as to be invisible under the microscope, have now been broken down. For in this zone the nuclei become amoeboid in form and move together in small groups, apparently without any hindrance from either cell boundaries or cytoplasm (Fig. 4). In these groupings, the nuclei are often in close apposition and, lower in this zone, some of them are considerably larger than the original ones while others are not. The irregular size increase as well as the greater number of nucleoli in these larger nuclei suggests that fusion occurs between apposed nuclei. This is made almost certain by the amounts of contained DNA as revealed through photometric measurements (Fig. 9 and Table I), made in both Zones III and IV.

TABLE I

Amount of desoyxribosenucleic acid (DNA)[1] in oogonia and oocytes of *Acanthocephala* ovary

Zone of end chamber	Number of nuclei (measured)	Mean diameter of nuclei in microns	Mean amount of DNA per nucleus	Multiple of DNA
1.....	10	7.6±0.9	2.9±0.3	Approximately 1
1.....	9	7.9±0.8	5.2±0.5	» 2
1.....	7	7.7±0.8	4.3±0.4	» 1.5
	5	8.4±0.7	3.3±0.4	» 1
3.....	9	9.5±0.8	5.6±0.4	» 2
	7	8.7±0.7	6.7±0.7	» 2
	8	9.4±1.0	8.6±0.9	» 3
	3	9.0	11.2	» 4
4.....	11	12.0±1.5	14.8±2.0	» 5
	3	13.6	17.5	» 6
	2	17.0	18.6	» 6
	3	18.5	26.0	» 9
	3	22.0	50.4	» 17
4.....	5	9.4±0.8	5.1±0.7	» 2
	9	12.0±1.4	2.0±0.25	» 0.7
Area in which	10	13.3±1.5	4.3±0.62	» 1.5
loss of DNA	3	15.2	1.5	» 0.5
can be noticed	4	19.3	5.9	» 2
	4	21.2	12.8	» 4
	2	24.6	47.0	» 15
	2	9.5	11.6	» 4
	3	6.8	3.3	» 1
	122 nuclei measured			

These show that the amounts of DNA carried by the nuclei of these zones differ greatly, the largest quantity found being 17 times as great as the smallest. Further, these values do not represent only exact multiples as they do in the liver of rats and mice (13, 16, 8) but also show a considerable range of intermediate values. These various amounts of DNA stand in some degree of correlation with the size of the nuclei (column 3 and 4, Table I) which may be additional evidence of a prior fusion.[2]

[1] In Arbitrary Units.

[2] We are aware that the correlation between nuclear size and amount of contained DNA must be regarded with caution. Our own findings in the germ cells of *Arvelius* demonstrate that the volume of nuclei may increase 16 times without a change in the amount of DNA (15) and that furthermore the tetraploid quantity of DNA may be carried by liver nuclei of the rat which are no larger than those containing only the diploid quantity (7).

It may be pointed out that fusion processes are not unprecedented in hemipteran gonads for they occur regularly in certain testicular lobes of several species of *Loxa* (14). In those, chromosome and centriole counts demonstrate conclusively that nuclei fuse with each other and the phenomenon is accompanied by a disappearance of cell walls and grouping of amoeboid nuclei which are strikingly similar to what is observed in the end chamber of the *Acanthocephala* ovary.

This is not to deny that endomitosis may also occur in some nuclei. The discovery, by Lison and Fautrez-Firlefyn (9), of accumulations and extrusion of DNA in the ovary of the crustacean *Artemia* suggests that a process similar to what has here been described in *Acanthocephala*, occurs there also. But these authors report quantities of DNA that constitute exact multiples (1:2:4 etc.) and interpret such results as the "sequel of successive waves of endopolyploidy." In *Acanthocephala* that would not account for the disparate quantities of DNA that are encountered.

In the last or most posterior region of the end chamber, Zone IV, the aggregation of nuclei in small groups, persists. But the size of individual nuclei in general shows a progressive increase, whereas the number in each group gradually decreases. In short, the tendency toward fusion continues in this last zone and this is again supported by the considerable increases in the amount of contained DNA.

But the outstanding feature in Zone IV is one which is only begun in Zone III. It takes the form of a running-together or laking of the Feulgen-positive material in each nucleus (Fig. 5 and 6). Subsequently one of two processes seems to occur. In the first, some of the accumulated DNA percolates through the nuclear membrane, gathering as a large drop in the cytoplasm outside. The gathering of this Feulgen-positive material usually occurs around a nucleolus—possibly a purely mechanical event—and, remarkably enough, this nucleolus usually makes its way out of the nucleus with the drop of DNA. The fact that it appears to lag behind in this process would indicate that the nucleolar extrusion does not occur without mechanical difficulty, but finally a spherical drop of Feulgen-positive material with a centrally placed nucleolus is encountered out in the cytoplasm (Figs. 6 and 7). Alternatively, but less commonly, the Feulgen-positive material is gathered around the inside of the nuclear periphery but fails to penetrate the membrane (Fig. 7). In such a case, the nucleus as a whole finally becomes perfectly spherical and collapses. The end result is again a drop of Feulgen-positive material and it has not been possible to distinguish between the two types in the final stages of droplet formation. The essential fact is that Feulgen-positive droplets

are now found free in the cytoplasm which itself is continuous through the zone and not restricted by any cell membranes.

In the posterior part of Zone IV these drops occur in considerably larger sizes which indicates that they run together when they come in contact with each other. Less easily explained is the fact that in the great majority of these drops in the lower part of the end chamber, the centrally located nucleolus has disappeared, leaving there an unstained space or vacuole. It is possible that this may be due to a disintegration and absorption of the nucleolus, but it may also result from its bodily extrusion from the DNA droplet. In Feulgen preparations counter stained with fast green—in every other respect exceptionally favorable for our study—a nucleolus thus extruded would be difficult to recognize for the adjacent cytoplasm of this region usually stains almost or quite as intensely with the fast green as does the nucleolus itself.

Although nuclei, from which all photometrically detectable Feulgen-positive material has been extruded, appear almost devoid of any contents except for an occasional nucleolus, there actually remain many threads or a fine network that extend through the nuclear space. This thready material appears to be Feulgen-negative in the great majority of cases. However, in some instances, later stages show a renewed but rather limited appearance of Feulgen-positive material in conjunction with such structure. Apparently this material again accumulates and goes out through the nuclear membrane. This later process does not resemble the bursting-out of a droplet as already described but is more akin to a seepage through a comparatively large area of the membrane (Fig. 8). Probably such material joins the droplets already formed and is then indistinguishable.

C. *Transfer to the egg.* Through the center of Zone IV, and reaching anteriorly as far as Zone III, extends a large column or core of homogeneous material in which no cellular structure is visible. This material stains strongly with fast green, is confluent laterally with the cytoplasm which surrounds the nuclear aggregates and is, indeed, cytologically indistinguishable from such cytoplasm. Generally speaking therefore, the disposition of this material in the end chamber is in the form of a tree whose branches are formed by the continuous cytoplasm extending peripherally between the many groups of nuclei (Fig. 1). Posteriorly the main column breaks up into a few small ones, one of which enters directly into the nearest, youngest egg while others take their course around this egg and connect with the next, older egg and even with the third much larger egg still farther toward the posterior of the egg string. The cytological evidence thus demonstrates a direct connection

between the general cytoplasm of the end chamber and the growing immature eggs.

The events in the end chamber might then be outlined as follows: The processes in Zone IV lead to final breakdown of cellular structures and to a contribution of the disintegration products to the central core or column of nutritive material that leads to the eggs. There is however a continuous replacement of nuclei and cytoplasm in this zone from the more anterior zones, the ultimate source of new cells being Zone I which is the only region of the end chamber where a normal mitosis and cell division occur.

This account implies that there is a streaming of material from the peripheral regions of the end chamber toward the main column and then to the developing eggs, a conclusion that was also held by earlier investigators (see for instance Gross (3)). There is indeed no reason to doubt such a transfer of substances and in haematoxylin preparations the narrow regions of the column usually show wavy longitudinal striations which indicate lines of flow (Fig. 1). But it need hardly be pointed out that such streaming must be extremely slow. The growth period of the eggs in coreid and pentatomid Hemiptera may last for weeks and the total number of eggs laid is comparatively small when compared with that of many other insects.

The breakdown of cell structures in Zones III and IV involves certain steps that might be summed up as follows: There is first a disintegration of cell membranes in Zone III which permits a gathering of nuclei in groups and their fusion with each other. In both Zones III and IV this is followed by a nuclear extrusion of DNA in the form of droplets which are then found free in the continuous cytoplasm surrounding the nuclear aggregations. Apparently the DNA, when free in the cytoplasm, disintegrates rapidly for in *Acanthocephala* Feulgen-positive droplets are very rarely found in the main column of nutritive material. But there is some variation in this respect, for in *Acanthocephala terminalis*, and *Spartocera*, as well as in certain other species such droplets are more often encountered in the central column of material.

The coreids thus furnish direct evidence that DNA or its derivatives are contributed to the nutritive materials carried by the egg. An addition of DNA or chromatin to the egg substance of various animals has been reported by several earlier investigators (see for instance (9, 12)) though the cytochemical evidence is, in most cases, not complete. It is an interesting fact, however, that in most other species the same end is attained in ways that are superficially quite different from those seen in the Hemiptera. That would seem to emphasize the fact that DNA or its breakdown products forms an im-

portant constituent of the nutrient materials of the egg and that if the basic structure of the gonad presents difficulty to its transfer into the egg, other ways are evolved to deliver it there.

DISCUSSION

The sparsity of Feulgen-positive droplets in the main stream of material that is transferred to the egg would seem to indicate their rather rapid disintegration.

The changes involved are open to a preliminary analysis involving the following steps:

1) Whereas in the normal nuclei in the anterior region of the end chamber the ratio of Feulgen-positive to methyl green-stained material is 1:1, the same two staining methods applied to the extruded droplets show a corresponding ratio of 3:1. This constitutes evidence that a depolymerization of DNA is in progress in the formation of the droplets (4, 5, 11).

2) Sections subjected to the PAS treatment show no red stain in the normal cells in the anterior region of the end chamber. However, the Feulgen-positive droplets which are extruded from the nuclei in Zones III and IV, show the typical red stain indicating the presence of polysaccharides. These are present in the form of small red granules disposed through the droplets. They are not demonstrable with ordinary techniques.

3) Sections treated with the enzyme DNAse show a marked decrease of Feulgen-positive material in the normal nuclei, as might be expected. However, the extruded droplets were affected by the enzyme to a much smaller degree. This difference is not easy to interpret. It is possible that the presence of the polysaccharides serves as a protective agent for the DNA, but it is not clear how this could be brought about.

To sum up, it is clear that in addition to the materials furnished by the regular follicle cells, the growing egg receives contributions from the end chamber. The latter comprise not only cytoplasm liberated by the breakdown of cell walls but also certain materials which are extruded from the nuclei. These nuclear extrusions in the form of droplets are at first strongly Feulgen-positive but undergo extensive changes before they are incorporated in the egg.

SUMMARY

1) The end chambers of the ovarial lobes in coreid Hemiptera contribute to the nutritive materials which are stored in the growing egg.

2) Such nutritive materials are derived from nuclear DNA as well as cytoplasm.

3) The processes involved include a breakdown of cell walls as well as an irregular fusion of nuclei and an extrusion of DNA in the form of droplets through the nuclear membrane.

4) Before the flow of such materials reaches the egg, the contents of the droplets undergo considerable alteration and become Feulgen-negative. The changes involve the formation of polysaccharides and some depolymerization of DNA, but still further alterations probably take place.

REFERENCES

1. Alfert, M., *J. Cell. Comp. Physiol.*, **36**, 381 (1950).
2. Caspersson, T., *Skand. Arch. Physiol.*, **73**, Suppl. 8, 1 (1936).
3. Gross, J., *Z. Wiss. Zoöl.*, **69**, 139 (1901).
4. Kurnick, N. B., *J. Gen. Physiol.*, **33**, 234 (1950).
5. Leuchtenberger, C., *Chromosoma*, **6**, 449 (1950).
6. Leuchtenberger, C., and Schrader, F., *Proc. Natl. Acad. Sci.*, **36**, 677 (1950).
7. —— *Biol. Bull.*, **101**, 130 (1951).
8. Leuchtenberger, C., Vendrely, R., and Vendrely, C., *Proc. Natl. Acad. Sci.*, **37**, 33 (1951).
9. Lison, L., and Fautrez-Firlefyn, N., *Nature*, **166**, 610 (1950).
10. Lison, L., and Pasteels, J., *Arch. Biol.*, **62**, 1 (1951).
11. Pollister, A. W., and Leuchtenberger, C., *Proc. Natl. Acad. Sci.*, **35**, 111 (1949).
12. Ries, E., *Z. Zellforsch. u. mikroskop. Anat.*, **16**, 314 (1932).
13. Ris, H., and Mirsky, A., *J. Gen. Physiol.*, **33**, 125 (1949).
14. Schrader, F., *J. Morphol.*, **76**, 157 (1945).
15. Schrader, F., and Leuchtenberger, C., *Exptl. Cell Res.*, **1**, 421 (1950).
16. Swift, H., *Physiol. Zoöl.*, **23**, 169 (1950).
17. Weber, H., Grundriss der Insektenkunde, 2nd ed., Fischer, Jena, 1949.

THE EFFECT OF VITAMIN B_{12} AND THE ANIMAL PROTEIN FACTOR ON THYROID-FED RATS, WITH SPECIAL REFERENCE TO LIVER CYTOLOGY

UNNE STENRAM

Histological Institute of the University of Lund, Sweden

Received June 10, 1951

Emerson (3) found that rats—given desiccated thyroid to increase the vitamin demands—on a diet with soybean meal as the only protein source and all known micronutritients included, gained less weight, if they were not given vitamin B_{12}. Ershoff (6) showed that, if the protein is given as vitamin-free casein, vitamin B_{12} is of no growth-stimulating value. Later (2, 20) it was found, in researches with pigs and chicks, that an animal protein factor feeding supplement (APF) is a still stronger growth-stimulant than vitamin B_{12}. At the same time Shive *et al.* (18) found that vitamin B_{12} can replace thymidine as a growth-factor for bacteria. It was postulated that the vitamin acts as an enzyme in the synthesis of thymidine and therefore of desoxyribonucleic acid too.

If vitamin B_{12} has the same function in higher animals it should be possible to study cytologically its qualitative and quantitative effect on the nucleic acid containing structures. Therefore Stern *et al.* (19) made a histological study on liver cells of rats which had and had not received vitamin B_{12}. The amount of nucleic acids was decided by subjectively evaluating the staining intensity with methylene blue. They found a more pronounced basophilia in the liver cell cytoplasm of the rats which had received vitamin B_{12}. The staining method employed, however, is unspecific and gives no information of the nucleic acid nature of the structures observed.

It was therefore thought desirable to study cytologically the effect of vitamin B_{12} and APF primarily on liver cells in thyroid-fed and normal rats.

MATERIAL AND METHODS

For the experiments a rat strain (*Mus norvegicus albinus*) was used that has been bred for many years in this institute. These rats are given crushed mixed grain and whole milk. The litters from which the experimental animals would be taken were reduced to 4 immediately after parturition to get similar growth conditions. From

each litter only one animal was taken for each experimental group. When 25 days old, the males were placed in special cages with glass walls and with a bottom of glass ribs in order to avoid accumulation of faeces and thus coprophagia.

The diet consisted of (6): 73.5 per cent sucrose, 22.0 per cent casein (washed three times with boiling alcohol, AB Vitrum, Sweden), and 4.5 per cent salts, pro analysi (10).

To 100 g of this mixture the following micronutritients were added (3): 1 mg thiamine, 2 mg riboflavin, 1 mg pyridoxine, 10 mg calcium pantothenate, 10 mg nicotinamide, 5 mg inositol, 30 mg p-aminobensoic acid, 100 mg choline chloride, 0.2 mg pteroylglutamic acid[1], 0.05 mg biotin, and 14.2 mg 2-methyl-1.4-naphtoquinone.

Every second day each rat was given 0.3 cc of the following mixture (6): 0.5 g α-tocopherol, 62.5 g peanut oil and 2.5 g vitamin AD-concentrate (corresponding to 50 IU A vitamin and 20 IU D vitamin per animal each day).

0.17 per cent thyreoid. sicc.[1] (containing 0.3 per cent organic iodine, AB Astra, Sweden) was mixed into the diets of group A and an equal amount of sucrose omitted.

Vitamin B 12[1] (crystallinized, "Hepagon," AB Astra, Sweden) was given by intramuscular injection of 0.3 γ per animal each day (groups A 2 and B 2).

APF (Animal Protein Factor Feeding Supplement 5, Lederle Lab.)[2] was mixed into the diets to an amount of 2.5 per cent and an equal quantity of sucrose omitted (groups A 3 and B 3).

The experimental groups were:

A. With thyroid powder: 1. Basic diet. 2. Basic diet + vitamin B_{12}. 3. Basic diet + APF.

B. Without thyroid powder: 1. Basic diet. 2. Basic diet + vitamin B_{12}. 3. Basic diet + APF.

The animals were weighed at the same hour every day. They were given tap water and the experimental diet ad libitum. After 21 days they were killed by decapitation. The liver and kidneys were weighed and pieces of both kidneys and the right and the left lobes of the liver were fixed in Carnoy's fluid and 10 per cent formol.

OBSERVATIONS

Weight gains: see table.

The animals often lost weight during the first days in the experimental cages. After 5 days they all showed regular weight gains and therefore the mean values were determined on the last 16 days.

The groups not receiving desiccated thyroid (group B 2) showed the same weight gains whether they received the supplement of vitamin B_{12} or APF (B 3) or no supplement at all (B 1). In the groups receiving thyroid (A) all animals gained less weight. Those receiving vitamin B_{12} (A 2) gained as little as those receiving no supplement at all (A 1). These two groups are signif-

[1] Kindly furnished by AB Astra, Sweden.
[2] Kindly furnished by Lederle Lab.

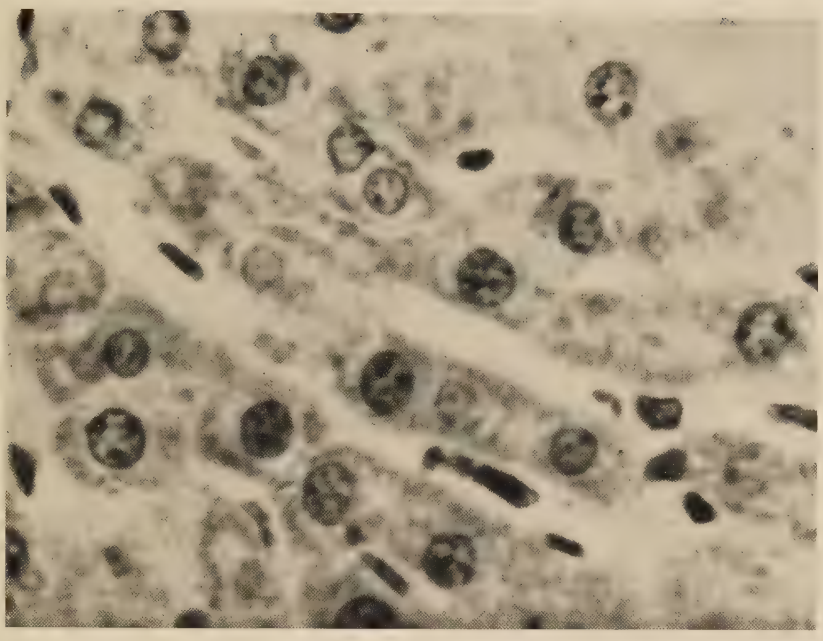


Fig. 1. Liver cells from an animal, which has received thyroid powder (group A). Gallocyanin-chrome alum stain pH 1.6. Carnoy fixation. Thickness of section 7 μ. Magnification ×1 000. Immersion objective N.A. 1.0 apochromat.

icantly different from group B as well as from the A 3 group (in both cases $P < 0.001$). Group A3 receiving thyroid and APF—is with great probability different from group B ($0.01 > P > 0.001$).

Liver: The liver weights per 100 g body weight (see table) of group A are significantly higher than those of group B ($P < 0.001$).

In sections, two quite different pictures have been obtained. In all livers belonging to group A (fig. 1) there is, with gallocyanin-chrome alum stain (pH 1.6), a faint, diffuse basophilia all over the cytoplasm. Palisade-like arranged rods of stronger basophilia are observed most often at right angles to the blood capillaries. In other cells these rods are irregularly scattered over the cytoplasm which may have been caused by unsuitable section-cutting.

In all livers belonging to group B (fig. 2) there are in the cytoplasm a few lumpy, very basophilic inclusions between which there is no basophilia at all. These "white" fields are probably occupied by glycogen, because when staining with Best's carmin no glycogen can be found in group A but it is clearly seen in group B.

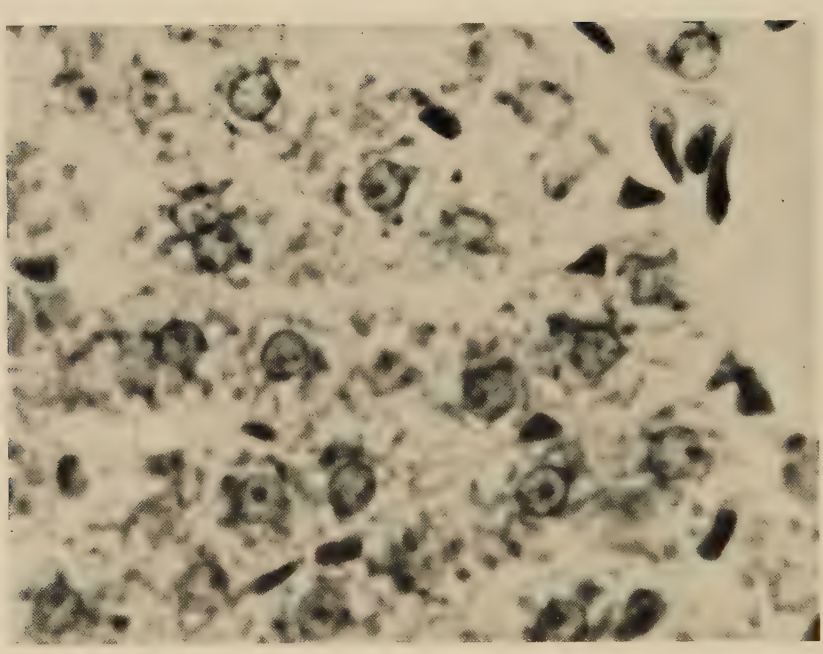


Fig. 2. Liver cells from an animal, which has not received thyroid powder (group B). (For technical details see fig. 1.)

With the Feulgen reaction there are well developed caps of positive material around the nucleoli in all sections.

With Sudan III all livers were found to have the same fat contents.

All observations were made on both the right and the left lobes of the livers.

Kidneys: The kidney weights per 100 g body weight (see table) of group A are significantly higher ($P < 0.001$) than those of group B. No study has yet been made to find out the histological basis of this difference.

Other organs: With routine staining no differences could be found in the histological pictures of some other organs (pancreas, spinal cord, cerebellum, cerebrum, thymus, adrenal, testis).

DISCUSSION

It is still very difficult to evaluate the often very discrepant results of investigations concerning the effect of growth promoting substances on thyroid-fed animals. This is partly due to the great variability in the diets used by the various workers. Ershoff, for example, has in several experiments (4–8)

TABLE I

Group	Weight gain[1]	Liver weight/100 g body weight		Kidney weight/100 g body weight	
A 1	1.6 g	6.20 g		1.41 g	
1	2.2	7.43		1.93	
1	1.7	6.25		1.74	
1	2.6	6.78		1.41	
2	2.1	7.15		1.75	
2	1.8	5.94		1.70	
2	2.0	6.30		1.45	
2	2.1	6.36		1.57	
3	2.5	6.14		1.53	
3	2.6	6.18		1.50	
3	3.0	6.89		1.59	
3	2.8	6.49	Mean:	1.65	Mean:
3	3.1	6.32	6.47 ± 0.12;	1.63	1.63 ± 0.05;
3	3.2	6.08	$s = 0.44$;	1.99	$s = 0.18$;
B 1	3.4	6.21		0.95	
1	3.4	5.78		1.06	
1	3.9	5.82		1.06	
1	3.6	4.82		0.84	
1	3.3	4.61		0.99	
2	3.2	5.23		0.99	
2	3.3	4.90		0.91	
3	2.9	5.31		0.93	
3	3.4	5.48		1.12	
3	4.1	5.29		1.12	
3	3.3	5.65	Mean:	1.08	Mean:
3	3.2	6.24	5.38 ± 0.15;	1.22	1.02 ± 0.03;
3	3.4	4.54	$s = 0.56$;	1.05	$s = 0.10$;

given his animals neither pteroylglutamic acid nor biotin, while others use these substances in varying amounts (1, 16). By changing the carbohydrate component (without changing its amount) of the food mixture quite different weight gains can be obtained (13). Different quantities and kinds of fats are used. Ershoff (5) has shown that an increase of the fat contents of the diet augments significantly the gains in body weight of hyperthyroid rats fed on a diet low in fat and containing casein as the dietary protein. The importance of the protein source has been mentioned in the introduction.

[1] The average body weight gain a day during the last 16 days of the experiment.

By varying the diet the intestinal bacterial flora is probably changed, which affects the vitamin state of the intestinal contents. Antibiotica have therefore been used in these investigations. The result is probably a profound change in the vitamin composition of the material, absorbed by the intestine, but further studies are here necessary.

The results reported here confirm the results of Ershoff (6) that vitamin B_{12} is of no value as a growth-promoting factor in the hyperthyroid rat, given casein as the dietary protein. Nor is it of value to rats on this diet without thyroid. APF, however, is of great benefit for the hyperthyroid rat.

In group B (those not receiving thyroid) the animals receiving APF or vitamin B_{12} did not grow better than their controls. Most workers, however, in their researches with pigs and chicks (2, 20) have found better weight gains in APF-fed animals. In preliminary unpublished experiments with chicks, Frölich (9) has not been able to confirm these results, what is probably explained by the more adequate basic diet which he used. The results of the present work are probably explained in the same way.

Previously the histological effect of thyroxin on liver cells has been studied by Schönholzer, for example (17). He reports that the coarse, basophilic inclusions of the well-nourished animal quite disappear after thyroxin administration. He has not observed the faintly basophilic, rodlike structures reported here. Such structures have been observed also after adrenalin administration and after acute bleeding (12). Opie (14) and Opie and Lavin (15) found similar structures after butter yellow feeding. These last mentioned workers have studied them with ultraviolet microscopy, ribonuclease and precipitation with lanthanum acetate and have shown that they have the characteristics of ribonucleic acids. This holds true also for the coarse, basophilic inclusions of their control material—structures certainly identical with the inclusions of the non-thyroid-fed animals of this investigation.

SUMMARY

1. Young male rats were given a basic diet with all protein in the form of alcohol-treated casein, and including all known micronutritients except vitamin B_{12}. One group was given this basic diet. Another group was given this diet + vitamin B_{12}. A third group was given the basic diet + animal protein factor. In each of these groups half of the animals were given desiccated thyroid.

2. Those animals given thyroid gained less weight than the others. If they were also given APF the weight gained was less than in those not receiving

thyroid but more than in those given thyroid but not APF and significantly different from the last mentioned group. All animals not receiving thyroid had the same weight gains.

3. The liver weights per 100 g body weight were significantly higher for the animals receiving desiccated thyroid.

4. The animals given thyroid had a faint, diffuse basophilia and more basophilic, palisade-like arranged rods in their livercell cytoplasm.

5. Those not given thyroid had coarse, strongly basophilic inclusions in their liver cell cytoplasm.

6. The kidney weights per 100 g body weight were significantly higher for the animals receiving thyroid.

REFERENCES

1. Betheil, J. J., and Lardy, A. H., *J. Nutrition*, **37**, 495 (1949).
2. Cunha, T. J., *et al.*, *Arch. Biochem.*, **23**, 324 (1949).
3. Emerson, G., *Proc. Soc. Exptl. Biol. Med.*, **70**, 392 (1949).
4. Ershoff, B., *Exptl. Med. and Surg.*, **6**, 438 (1948).
5. —— *J. Nutrition*, **39**, 259 (1949).
6. —— *Proc. Soc. Exptl. Biol. Med.*, **71**, 209 (1949).
7. —— *Ibid.*, **73**, 459 (1950).
8. —— *Ibid.*, **74**, 391 (1950).
9. Frölich, A., Personal communication.
10. Jones, J. H., and Foster, C., *J. Nutrition*, **24**, 245 (1942).
11. Kellaway, P. E., *et al.*, *Endocrinologia*, **36**, 272 (1945).
12. Lagerstedt, S., and Stenram, U. (In press.)
13. Lewis, U. J., *et al.*, *Proc. Soc. Exptl. Biol. Med.*, **74**, 568 (1950).
14. Opie, E., *J. Exptl. Med.*, **84**, 91 (1946).
15. Opie, E., and Lavin, G., *Ibid.*, **84**, 107 (1946).
16. Schaefer, A. E., *et al.*, *J. Nutrition*, **40**, 95 (1950).
17. Schönholzer, G., *Beitr. Pathol. Anat. Ziegler*, **97**, 526 (1936).
18. Shive, W., *et al.*, *J. Am. Chem. Soc.*, **70**, 2614 (1948).
19. Stern, J., *et al.*, *Proc. Soc. Exptl. Biol. Med.*, **70**, 551 (1949).
20. Stokstad, E. L. R., and Jukes, T. H., *Ibid.*, **73**, 523 (1950).

PHOSPHATIDE COMPOSITION IN DIFFERENT LIVER CELL FRACTIONS[1]

CELIA LEVINE[2] and ERWIN CHARGAFF

Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York

Received June 15, 1951

The understanding of the different patterns of composition that form the basis of the specificity of cells and of their structural elements requires a deeper insight into the types of lipids taking part in their architecture. Aside from the preparatory approach, which is necessarily wasteful and unsuitable for analytical applications, there exists no method that permits the complete mapping of the distribution of individual lipids in a tissue, especially when small quantities are to be surveyed. The most desirable solution of this problem would appear to lie in the application of procedures such as partition chromatography, perhaps coupled with the use of ion exchange resins, that would result in the quantitative separation of the lipids from each other; but this is hardly feasible at present. Even if it is assumed that the different phosphatides occur in the cell as monomers, and there is no real justification of such an assumption, the lack of a sufficient number of well characterized reference compounds would deprive these separation procedures of much of their value.

An orienting approach to the problem of characterization of lipid mixtures could be based on the separation and quantitative estimation of some of their distinctive constituents. The principal constituents of the group of phosphatides with which this paper deals are fatty acids, polyhydric alcohols (glycerol, *meso*-inositol), and aminoalcohols or their derivatives (ethanolamine, serine, choline, etc.). Attempts at the quantitative characterization of mixed lipids through group determination of constituents have frequently been made in the past, both in this laboratory (6, 11, 13, 14) and by other workers (1, 16, 20, 21, 32). The most detailed examination has recently been carried out by Brante (5).

[1] This work was supported by grants from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council and from the Life Insurance Medical Research Fund.

[2] This report is from a dissertation submitted by Celia Levine in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University, New York.

Within the group of phosphatides, a survey of their nitrogenous components appears to be particularly desirable, since it may be assumed that many of the biological functions of these lipids are connected with their polar characteristics. (Compare the discussion in (7)).

We have recently described methods, employing paper chromatography, for the separation and quantitative estimation in minute amounts of several nitrogenous phosphatide constituents, *viz.* ethanolamine, choline, and serine, and the application of these procedures to the study of a series of phosphatide fractions from beef brain (25). The present communication presents a survey of the composition of the phosphatides isolated from different cellular elements of rat liver, namely the nuclei and several particulate or submicroscopic cytoplasmic fractions. Previous work in this field and some of the implications of our findings will be considered later in this paper.

EXPERIMENTAL

Analytical Methods

Phosphorus was determined colorimetrically (23) and total nitrogen by the Kjeldahl procedure. For the analysis of their nitrogenous constituents the lipid samples were hydrolyzed in a 6 N solution of gaseous HCl in anhydrous methanol, a procedure introduced into lipid chemistry a long time ago (3, 28). The mixture was refluxed for three hours, the methanol removed *in vacuo* and replaced by the same volume of water. After having been heated to boiling for one hour under a reflux, the hydrolysate was cooled, repeatedly extracted with ether, filtered through "Celite," and evaporated to dryness *in vacuo* at a temperature below 40°, with the repeated addition of small amounts of water. The residue, dried over P_2O_5 and NaOH in a vacuum, was then adjusted for chromatography. The estimations of serine, ethanolamine, and choline were carried out by the chromatographic procedures described before (25). The micro electrophoresis apparatus used was a Kern instrument, type LK 30[1]. The absorption spectra were determined in a Beckman photoelectric quartz spectrophotometer.

Starting Material

The animals used were male rats of the Sherman strain, each weighing about 300 g. The preparation of nuclear material from liver made use of procedures employing 5 per cent citric acid which are described in the literature (26, 27). The white nuclei thus obtained after eight washings with citric acid gave good microscopic pictures and appeared free of cytoplasmic debris. The material isolated from 12 livers was suspended in 100 cc of citric acid, and aliquots were removed for microscopic examination. After repeated dialysis against fresh portions of ice-cold physiological saline, the suspension was freed of water in the frozen state in a vacuum. The

[1] We are grateful to Miss V. Van Veen, Kern Company, New York, for placing this instrument at our disposal.

weight of the dry nuclear preparation was 1.4 g (corrected for the NaCl content which was determined by a titrimetric chloride determination (29)).

The isolation of cytoplasmic fractions from 10 rat livers by differential centrifugation in 0.88 M sucrose solution followed, with a few modifications, previously published procedures (18, 22). The following fractions were collected: 1) Fraction Sd-24 (mitochondria); 2) Fraction Sp-24 (washings of mitochondria); 3) Fraction Sd-31 (microsomes or submicroscopic particles); 4) Fraction Sp-31 (non-sedimentable cytoplasmic material).[1] After the last centrifugation cycle at $1\,900 \times g$ (5 minutes), to remove coarse material, the supernatant was subjected to sedimentation at $24\,000 \times g$ for 30 minutes, when the crude mitochondrial fraction was separated from the supernatant containing the less easily sedimentable cell fractions. The sediment was washed with 100 cc of 0.88 M sucrose and recentrifuged at the same speed, yielding Sd-24 and a supernatant containing Sp-24. Fraction Sd-24 behaved on microscopic examination similar to preparations of mitochondria described in the literature (18). Both fractions were recovered by dialysis against distilled water and evaporation in a vacuum in the frozen state; Sd-24 weighed 1.38 g, Sp-24 0.63 g.

The reddish supernatant from the first sedimentation of mitochondria was subjected to centrifugation at $31\,000 \times g$ for three hours in the multispeed attachment of the refrigerated International centrifuge. The red translucent pellet was suspended in 100 cc of 0.88 M sucrose and dialyzed and recovered in the frozen state. The yield of Fraction Sd-31 was 0.48 g.

The almost clear supernatant of this fraction, when subjected to dialysis and pervaporation, deposited a voluminous precipitate which was collected by centrifugation at $1900 \times g$ for 30 minutes. This material, Fraction Sp-31, weighed 2.63 g. Another product, weighing 2.93 g, remained in solution. It was not further examined, since it yielded less than 1 per cent of material soluble in chloroform.

The lipids of unfractionated rat liver also were examined for purposes of comparison. The tissues were, as in all other cases, removed from the anesthetized animals, that had fasted for 18 hours, after perfusion with saline. Six livers, weighing 57 g, were homogenized in 300 cc of physiol. saline and dried in the frozen state. The weight of the dry tissue powder, corrected for the NaCl content, was 11.9 g.

Lipid Fractions

For the extraction of lipids, exactly weighed amounts of the dry preparations were treated at 60° with absolute ethanol-ether (3 : 1, by volume) for one hour in a nitrogen atmosphere. The residues were again extracted with chloroform-methanol (1:2) at 80° for one hour under N_2. The extracts thus obtained from each cellular fraction were combined and evaporated *in vacuo*. The residues were treated with chloroform, freed of undissolved material by filtration, and adjusted to known volumes with chloroform.[2] Aliquots of the solutions were taken to dryness, in order

[1] We prefer to refer to these fractions in terms of their isolation characteristics rather than by cytological designations. Sd and Sp stand for sediment and supernatant respectively; the figures, when multiplied with $1000 \times g$, indicate the centrifugal force employed.

[2] In the case of the nuclear lipids, an additional purification step consisted in three extractions of the chloroform solution with 10 per cent aqueous sodium chloride.

to determine the yield and to test for solubility in petroleum ether. The remainder was recovered by evaporation and intimately suspended in 50 to 100 cc of water. The suspensions were, after dialysis against running tap water for 24 hours and against ice-cold distilled water for 12 hours, frozen and evaporated in a vacuum. The lipids then were again dissolved in chloroform to a known volume and aliquots used for the various analyses recorded in Tables I and II.

RESULTS

Solubility.—The lipids extracted from the preparation of nuclear material were found to be insoluble in cold and in warm petroleum ether (boiling point 30–60°). Petroleum ether extracts of this lipid fraction neither left a visible residue nor did they contain phosphorus. The lipids from Fractions Sd-24 and Sp-24, i.e. the mitochondrial material, behaved similarly. Fraction Sd-31, essentially consisting of submicroscopic particles, yielded a lipid preparation that was completely soluble in cold petroleum ether. The major part of the lipids isolated from Fraction Sp-31, derived from the non-sedimentable portion of the cytoplasmic extract, was soluble in petroleum ether. A minor fraction of this lipid preparation, however, resembled in solubility the nuclear and the mitochondrial lipids. Total rat liver tissue yielded lipids that were completely soluble in petroleum ether at room temperature.

Ultraviolet Absorption.—The absorption spectra in the ultraviolet of the

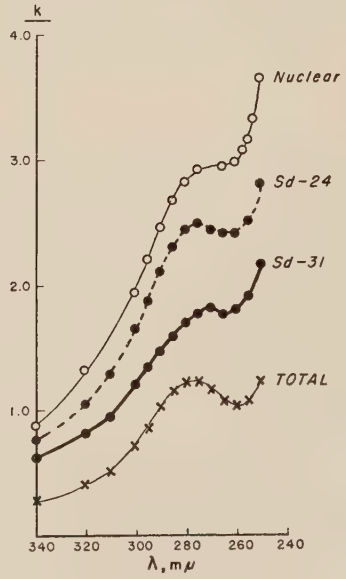


Fig. 1. Ultraviolet absorption of lipid preparations from rat liver fractions (dissolved in chloroform). k, the specific extinction, $= E/cd$, where $E = \log I_0/I$; c = concentration in g per liter; d = thickness of absorbing layer in cm.

TABLE I

Lipids from Rat Liver Fractions.

Starting Material	Lipids[1] per cent of starting material	Composition of purified lipids[2]		
		N per cent	P per cent	N:P
Total liver	19.3	1.38	2.80	1.09
Nuclear material	10.9		3.09	
Sd-24	13.7	1.44	2.86	1.11
Sp-24	18.9	1.58	2.58	1.36
Sd-31	29.1	1.57	2.80	1.24
Sp-31	13.5	1.12	2.35	1.05

TABLE II

Distribution of Nitrogenous Constituents in Hydrolysates of Lipids from Rat Liver Fractions.

Lipid Source	Recovered in hydrolysate		Moles of constituent found per mole of nitrogen subjected to hydrolysis				Moles of N accounted for per mole of total N in hydrolysate	Moles of constituent found per mole of phosphorus in hydrolysate				Molar ratio (Moles of constituent per mole of choline)	
	P	N											
	% of original	% of original	Choline	Ethanolamine	Serine	Total		Choline	Ethanolamine	Serine	Total	Ethanolamine	Seri
Total liver	98	93	0.45	0.29	0.07	0.81	0.88	0.50	0.32	0.08	0.90	0.64	0.1
Nuclear material	101							0.55	0.55	0.10	1.20	1.0	0.1
Sd-24	85	89	0.49	0.39	0.08	0.96	1.07	0.64	0.51	0.11	1.26	0.79	0.1
Sp-24	87		0.47	0.30	0.08	0.85		0.74	0.46	0.12	1.32	0.63	0.1
Sd-31	94	99	0.55	0.35	0.05	0.95	0.97	0.73	0.46	0.07	1.26	0.64	0.0
Sp-31	103	99	0.64	0.27	0.06	0.97	0.97	0.67	0.28	0.06	1.01	0.42	0.0

lipid preparations from total liver, nuclear material, and Fractions Sd-24 and Sd-31, examined as solutions in chloroform (rectified by distillation), are illustrated in Fig. 1. The clearest band was exhibited by the total liver lipids, with a maximum at 277, a minimum at 260 mμ. The nuclear lipids

[1] These figures are based on the weight of the chloroform-soluble lipids obtained by the extraction of the tissue fraction with ethanol-ether (3:1) and chloroform-methanol (1:2). In the case of the nuclear lipids, the loss in weight of the nuclear material after extraction under the same conditions was used.

[2] Chloroform-soluble lipids after dialysis, etc., as described in the text.

showed a plateau around 275 mμ. The lipids from Sd-24 and Sd-31 gave rather similar spectra, differing mainly in the height of extinction, which resembled the spectrum previously described for the lipids of the thromboplastic protein of beef lung (10).

Electrophoresis.—The electrophoretic behavior of one lipid preparation, namely that from Fraction Sd-24, also was examined. A 1 per cent suspension in 0.02 M phosphate buffer (containing 0.15 M NaCl) of pH 7.1 exhibited two components with descending mobilities of -15×10^{-5} and -23×10^{-5} cm^2 per volt per second respectively. Computations of the concentration from the interferometer patterns showed the two components to be present in about equal amounts. If it is assumed that the lipids containing serine and ethanolamine migrated together as the faster component, the electrophoretic findings are in agreement with the analytical results presented in Table II.

Composition.—Table I summarizes data on the composition of the intact lipid fractions. The distribution of nitrogenous constituents in their hydrolysates is presented in Table II. No novel lipid constituents (12, 25) were encountered, with the exception of the lipid preparation from Sp-31 which contained a minor fast-moving component reacting with ninhydrin. Its chromatographic behavior in *n*-butanol-diethylene glycol-water (4:1:1) resembled that of a fraction occasionally found in beef brain phosphatides (25); its R_F value was 0.48 in experiments in which the corresponding figures for serine and ethanolamine were 0.22 and 0.37 respectively. With serine employed as the colorimetric standard, the concentration of this component corresponded to 3.6 per cent of the N recovered in the hydrolysate.

DISCUSSION

The development of methods for the isolation of distinct cellular elements doubtless represents, at least in certain respects, an advance towards a better understanding of the chemistry of the cell. It must, however, be realized that, if the preservation of morphological features is to be the main goal, the conclusiveness of the chemical results may suffer. The application of the art of embalming to a cell component may afford a morphologically correct preparation and still involve a severe distortion of its chemical pattern, quite apart from the fact that, for instance in the case of nuclei, an isolated nucleus has been deprived of one of its most important characteristics, namely, to be surrounded by the cytoplasm.

The method for the isolation of cell nuclei (26, 27) used in the present study was adopted, because it was felt that, for purposes of a comparative

investigation of lipid composition, the nuclear and cytoplasmic elements should be as free of each other as could be accomplished. The isolation of the cytoplasmic fractions was achieved by relatively mild procedures.

The lipids of cell nuclei have been examined in a few instances (17, 30, 33), as have, more frequently, those of several particulate fractions of the liver cytoplasm, sometimes designated as mitochondria and as submicroscopic particles or microsomes (2, 4, 6, 15, 19, 24, 31). The lipids associated with a related cytoplasmic fraction, the thromboplastic protein of beef lung, have been studied in particular detail (7, 8, 10, 11). Lipids from preparations of the thromboplastic proteins derived from other tissues also have been described (9). A comparison of the figures for total lipid contents reported in previous investigations and in the present study appears pointless, since the procedures of preparation, extraction, purification, and analysis varied widely in many instances.

A striking property of the lipids isolated from certain cellular elements of the hepatic cell, to which little attention seems to have been paid, is the difference in solubility exhibited by them as compared with the lipids from unfractionated liver tissue. Stoneburg (30) mentioned the insolubility of the nuclear lipids in ether. The lipids from a preparation of rabbit liver mitochondria were found, in this laboratory, to contain a high proportion of material insoluble in petroleum ether (6). Similar observations were made in the present study in the case of the lipids of the nuclear material and of the mitochondrial fractions Sd-24 and Sp-24. The lipids associated with the small particles Sd-31 and with the non-sedimentable fraction Sp-31, on the other hand, did not differ in solubility from those of total liver tissue. Unfortunately, no cogent reason of this peculiarity can as yet be offered. The observed differences in the composition of the several lipid fractions are not sufficiently large to account for such discrepancy. But it may be assumed that the physical properties of a mixture or of a complex association of lipids will be governed not only by the characteristics of their ultimate constituents, but also by interactions between the molecules that are difficult to define in terms of our present knowledge. Moreover, it may be fallacious to consider cellular lipids as existing entirely in the form of monomers; and differences in the degree of polymerization would probably entail differences in solubility.

The quantitative findings on the distribution of nitrogenous constituents, summarized in Table II, showed the proportions of bases to be essentially the same in the lipids of total liver tissue and in those of the submicroscopic particle fraction Sd-31. The nuclear lipids, however, and to a lesser degree

those of the mitochondrial preparation Sd-24, had a relatively higher ethanolamine content. No major unidentified nitrogenous lipid component can have been present in the lipids of the various liver fractions, as shown by the proportion of nitrogen accounted for as individual constituents determined in the hydrolysates. The high N:P ratio found in some of the intact lipid preparations (Table I), which has also been observed by other workers (31), was reflected in the proportions of total moles of nitrogenous constituents per mole of phosphorus (Table II). Whether this relative lack of phosphorus is indicative of the existence of lipid polymers will have to be decided in later work.

SUMMARY

The isolation of lipid preparations from several cellular fractions of rat liver (nuclei, mitochondria, submicroscopic particles, non-sedimentable portion) and from total liver tissue is described. Recently developed micro methods for the separation and quantitative estimation of nitrogenous lipid constituents were applied to the determination of the distribution of choline, ethanolamine, and serine in the hydrolysates of the different lipid fractions. These bases accounted for practically all the lipid nitrogen. Certain characteristics of the lipids with respect to solubility, ultraviolet absorption, and electrophoretic behavior are pointed out and some of the implications of the findings are discussed.

REFERENCES

1. Artom, C., and Fishman, W. H., *J. Biol. Chem.*, **148**, 405 (1943).
2. Barnum, C. P., and Huseby, R. A., *Arch. Biochem.*, **19**, 17 (1948).
3. Baskoff, A., *Z. physiol. Chem.*, **57**, 395 (1908).
4. Bensley, R. R., *Science*, **96**, 389 (1942).
5. Brante, G., *Acta Physiol. Scand.*, **18**, Suppl. 63 (1949).
6. Chargaff, E., *J. Biol. Chem.*, **142**, 491 (1942).
7. —— *Advances in Protein, Chem.* **1**, 1 (1944).
8. —— *Advances in Enzymol.*, **5**, 31 (1945).
9. —— *J. Biol. Chem.*, **161**, 389 (1945).
10. —— *ibid.*, **173**, 253 (1948).
11. Chargaff, E., Bendich, A., and Cohen, S. S., *ibid.*, **156**, 161 (1944).
12. Chargaff, E., Levine, C., and Green, C., *ibid.*, **175**, 67 (1948).
13. Chargaff, E., Ziff, M., and Rittenberg, D., *ibid.*, **138**, 439 (1941).
14. —— *ibid.*, **144**, 343 (1942).
15. Claude, A., *J. Exptl. Med.*, **84**, 51, 61 (1946).
16. Hack, M. H., *J. Biol. Chem.*, **169**, 137 (1947).
17. Haven, F. L., and Levy, S. R., *Cancer Research*, **2**, 797 (1942).
18. Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., *J. Biol. Chem.*, **172**, 619 (1948).
19. Huseby, R. A., and Barnum, C. P., *Arch. Biochem.*, **26**, 187 (1950).
20. Johnson, A. C., McNabb, A. R., and Rossiter, R. J., *Biochem. J.*, **43**, 573 (1948).
21. Kaucher, M., Galbraith, H., Button, V., and Williams, H. H., *Arch. Biochem.* **3**, 203 (1943–44).

22. Kennedy, E. P., and Lehninger, A. L., *J. Biol. Chem.*, **179**, 957 (1949).
23. King, E. J., *Biochem. J.*, **26**, 292 (1932).
24. Kretchmer, N., and Barnum, C. P., *Arch. Biochem. Biophys.*, **31**, 141 (1951).
25. Levine, C., and Chargaff, E., *J. Biol. Chem.*, **192**, 465, 481 (1951).
26. Marshak, A., *Science*, **92**, 461 (1940).
27. —— *J. Gen. Physiol.*, **25**, 275 (1941).
28. Rollett, A., *Z. physiol. Chem.*, **61**, 210 (1909).
29. Schales, O., and Schales, S. S., *J. Biol. Chem.* **140**, 879 (1941).
30. Stoneburg, C. A., *ibid.*, **129**, 189 (1939).
31. Swanson, M. A., and Artom, C., *ibid.*, **187**, 281 (1950).
32. Taurog, A., Entenman, C., Fries, B. A., and Chaikoff, I. L., *ibid.*, **155**, 19 (1944).
33. Williams, H. H., Kaucher, M., Richards, A. J., Moyer, E. Z., and Sharpless, G. R., *ibid.*, **160**, 227 (1945).

THE TWO TYPES OF NUCLEOPROTEINS DURING MITOSIS

W. JACOBSON[1] and M. WEBB

From the Strangeways' Research Laboratory, Cambridge, Great Britain

Received July 12, 1951

In a study of the action of folic acid antagonists, smears of bone marrow cells and a variety of other cells, grown *in vitro*, were stained by the May-Grunwald and Giemsa technique, to demonstrate the cytoplasmic basophilia. The latter, which is characterised by a bright blue colouration, has been shown by Brachet (1, 2, 3), Wislocki and Dempsey (43), White (40) and others to be due to ribonucleoprotein. It was noticed that in dividing cells, meta- and anaphase chromosomes stained blue-black in marked contrast to the purple-red colour of pro- and telophase chromosomes.

Evidence will be submitted in this paper to show that ribo- and deoxyribonucleoproteins of the cell are stained respectively blue and purple-red by the May-Grunwald and Giemsa method. Also it will be shown that meta- and anaphase chromosomes carry a charge of ribonucleoprotein, and that during anaphase some of this material is transferred to the cytoplasm (18 a, 19).

MATERIALS AND METHODS

Smears were made of normal and leukaemic human and mouse material. The mouse leukaemia was a transplantable, acute lymphoblastic leukaemia in a hybrid FAK_1-strain. The following tissues were investigated by the smear technique: bone-marrow, lymphnodes, spleen, liver, kidney and intestine.

The tissues, cultivated *in vitro*, were all grown according to the hanging drop method. The following tissues were explanted: chick embryo osteoblasts, fibroblasts of the sclerotic and heart, and also spleen, spinal cord, skin and liver. As the chick osteoblasts and fibroblasts provided the greatest number of dividing cells, most of the results recorded were obtained with these tissues; but the observations were generally checked on other types of cells. More than 300 cultures were used in the course of this investigation.

STAINING METHODS

A. *The May-Grunwald and Giemsa Method*

1) *Staining of cell 'smears.'* The following standard technique was employed when dealing with cells spread in single layers on slides, as e.g. blood, bone marrow, tumour cells, or smears prepared from the cut surface of any organ.

[1] Sir Halley Stewart Research Fellow.

a) Immediately after preparation, the smears were placed in absolute methanol. This ensured instantaneous fixation of the nucleoproteins and also allowed a number of smears to be prepared, since the cells may be kept in methanol for several hours without observable loss in staining capacity.

b) They were transferred directly to undiluted May-Grunwald's solution and stained for 8 minutes. Blood smears required only 4 minutes.

c) They were drained and stained with Giemsa's solution, diluted with glass distilled water (1:10 or 1:15) for 15–20 minutes.

d) The excess dye was washed off with glass distilled water and the smears were dried.

2) *Staining of cells grown in vitro.* a) The tissue cultures were fixed in absolute methanol for not less than 5 minutes; b) They were placed into May-Grunwald's dye for 10 minutes, and then c) transferred directly into Giemsa's dye, diluted 1:15 with glass distilled water, and stained for 20 minutes. d) The cultures were washed rapidly with glass distilled water for about 10–20 seconds by agitating, in order to remove the excess dye. e) The water was replaced by acetone which was changed after 30 seconds. While in acetone, the cultures were shaken in order to obtain rapid dehydration. After about 1 minute in acetone the cultures were placed for 1 minute in a mixture of equal parts of acetone and xylene. f) They were transferred to xylene which was changed twice. g) D.P.X. or a similar synthetic mounting medium may be used. In Canada balsam the dyes are apt to fade.

The results were as follows:—The cytoplasm of many cell types and the nucleoli of all cells were stained blue. The chromatin of the resting nuclei was stained purple-red.

Techniques 1) and 2) gave consistant results if the following precautions were observed:—

a) Instantaneous fixation was essential. When smears of cells were allowed to dry in air before fixation (as is common practice) it was noted that e.g. the chromosomes became indistinct and swollen, and some of the blue staining material was lost from the cytoplasm. Therefore, each smear was placed into methanol without any delay whatsoever. This gave excellent fixation, except in some parts of the smear where the cells were unevenly spread.

When placing tissue cultures in methanol, it was found to be essential to move them rapidly in the fixative; otherwise the small amount of liquid covering the cells formed a coat of amorphous precipitate which delayed the penetration of methanol into the cells and obscured the stained preparation. The fixed tissues may be kept in methanol for several hours.

Absolute methanol of analytical or equivalent standard of purity was used. B.D.H. Methanol ('Solvent') gave very good results, but so-called 'purified' methanol was less satisfactory.

b) The May-Grunwald dye used was either the ready-made solution (Gurr's) or it was prepared (more economically) from the dry powder (Gurr's) by the following method:—

To 100 ml absolute methanol at 50° C 0.3 gm of the dye was added in minute portions with vigorous shaking. The solution was allowed to stand for 24 hours and then filtered (42).

c) The diluted Giemsa solution was always prepared freshly. In some cases it was replaced by Leishman's stain in the same concentration with good results.

d) If, during dehydration of the tissue cultures, shaking of the acetone was omitted, less satisfactory results were obtained.

B. *Methylgreen Pyronin*

This method was used as a control, since it was known to stain deoxyribonucleoproteins greenish-blue and ribonucleoproteins red (3, 40). The smears and tissue cultures, fixed with methanol, were stained for one hour in a mixture of methylgreen and pyronin, according to White's (40) modification of Unna-Pappenheim's solution. The composition of the mixture used was as follows:—

0.3 gm pyronin G (B.D.H. or Geigy), 0.7 gm methylgreen (Anachemia, C.I. 685), 20 ml glycerol, 2.5 ml absolute ethanol, and 0.5 per cent phenol in distilled water to 100 ml.

After staining for one hour the smears were washed with glass distilled water, blotted and dried. The tissue cultures were rapidly dehydrated through absolute ethanol, cleared in xylene and mounted in D.P.X.

Some cultures were stained with pyronin only. The same procedure was used as above.

C. *Feulgen Staining*

Feulgen's method served as a control for the staining of deoxyribonucleoproteins. Good results were obtained with the cultures after 7 minutes hydrolysis in 1 *N* hydrochloric acid at 60° C.

PREPARATION OF THE ENZYMES

Ribonuclease was isolated from beef pancreas by the methods of Kunitz (22) and McDonald (25). Both preparations were active against yeast ribonucleic acid and were free from phosphatase, nucleophosphatase, lipase, proteinase, adenine and guanine deaminase and deoxyribonuclease activities.

Deoxyribonuclease was isolated from pancreas according to the method of McCarty (24) and was purified by repeated fractionation with ammonium sulphate (28). The preparation was free from adenine and guanine deaminase, nucleotidase and phosphatase activity. It had a weak proteolytic activity when examined against a casein substrate, but exhibited no hydrolytic activity on Witte peptone.

The presence of the proteinase impurity in deoxyribonuclease was noted by McCarty (24) who suggested the use of gelatin as a stabiliser for dilute solutions of the enzyme. Even in the presence of gelatin (0.1 per cent), however, cells treated with the deoxyribonuclease solution (0.05 per cent) showed marked proteolytic digestion. However it has been shown that trypsin, chymotrypsin and chymotrypsinogen are inhibited by hydroxylamine and semicarbazide (32) and by several thiol compounds including cysteine and glutathione (17, 29). These substances are without effect upon the activity of deoxyribonuclease (16). Thus it was possible to obtain a deoxyribonuclease solution free from any trace of proteolytic activity by the addition of any of the above substances. In practice cysteine and hydroxylamine in concentrations of 0.05 *M* proved suitable and were used in the digestion experiments.

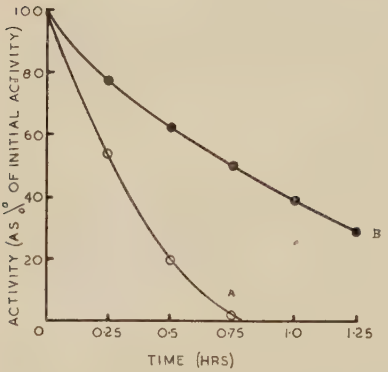


Fig. 1. Inactivation of a 0.02 per cent solution of deoxyribonuclease in 0.05 M aqueous sodium acetate, pH 6.8 at 18°C, alone (curve A), and in the presence of 0.05 M hydroxylamine as proteinase inhibitor (curve B). Immediately after preparation and at 15 minute intervals thereafter, aliquots (1 ml) of the enzyme solutions were assayed for activity against 0.2 per cent thymus deoxyribonucleic acid (2 ml) at pH 7, according to (16).

The enzyme was dissolved in water and the solution diluted with an equal volume of either 0.1 *M* cysteine hydrochloride or 0.1 *M* hydroxylamine hydrochloride in 0.1 *M* sodium acetate at pH 6.8. The mixture was brought to 37° C, then diluted with isotonic veronal buffer and used for the digestion experiments.

Inhibition of the contaminating proteinase not only prevented the proteolytic digestion of cells in tissue cultures, but also markedly increased the stability of dilute aqueous solutions of deoxyribonuclease. For example, a 0.02 per cent solution of deoxyribonuclease lost c. 80 per cent of its initial activity against thymus deoxyribonucleic acid when kept for 30 minutes at room temperature (18° C), whereas the loss in activity in the presence of hydroxylamine amounted to c. 25 per cent (Text-figure 1). It is apparent from Text-figure 1, however, that even under conditions of complete inhibition of the proteinase, aqueous solutions of deoxyribonuclease are relatively unstable and lose c. 70 per cent of their initial activity in 75 minutes at 18° C.

Although activation with magnesium is essential for the action of deoxyribonuclease on deoxyribonucleic acids (14, 24) the action of the enzyme on cells in tissue cultures occurred in the absence of added magnesium ions. This agrees with the observations of Gilbert, Overend and Webb (16), that the degradation of thymus deoxyribonucleohistone with deoxyribonuclease occurs in the absence of ionic magnesium. Catcheside and Holmes (7) also found that deoxyribonuclease, prepared from pancreas, removes the Feulgen positive bands from salivary gland chromosomes of drosophilia, irrespective of whether abundant magnesium ions are present or not.

OBSERVATION ON CELLS AND NUCLEOPROTEINS STAINED BY THE MAY-GRUNWALD AND GIEMSA METHOD

I. *Cells*

A. *Resting cells. Cytoplasm.* Undifferentiated cells (e.g. fibroblasts, myeloblasts and others) contain considerable amounts of intensively blue staining material which is evenly distributed throughout the whole of the cytoplasm

in the form of fine particles. These are present in such large amounts that, under low power, the cytoplasm appears bright blue. The small cytoplasmic areas between the blue particles are colourless (Fig. 2). When the cells are exposed to the May-Grunwald dye beyond the stated times, some undifferentiated cells, e.g. osteoblasts and fibroblasts, showed pink stained, round or slightly elongated particles between the blue stained cytoplasmic areas (Fig. 3). Whether these are mitochondria cannot be stated. The cytoplasm of mature cells varies considerably in the amount of blue staining material present, as has been shown previously by Wislocki and Dempsey (43), Thorell (37) and White (40) for the bone marrow. The myeloblasts and erythroblasts lose all their cytoplasmic basophilia in the course of differentiation into leucocytes and red blood cells.

The cytoplasm of histiocytes, on the other hand, contains a large amount of intensely blue staining material. Liver cells and those lining the proximal convoluted tubules of the kidney contain scattered blue staining particles of varying sizes in their cytoplasm.

Nuclei. In all nuclei a delicate pattern of irregular, purple-red stained strands corresponding to the "chromatin network" is observed (Fig. 2). The strands are so fine that it is uncertain whether they form an actual network or whether they are merely fine threads which cross or touch each other. Often the impression is gained that they are individual units. Fell and Hughes (13) observed by phase contrast microscopy a similar pattern in living cell nuclei. The fine strands appear more distinct when stained by the May-Grunwald and Giemsa technique than by Feulgen's method or methylgreen.

The Nucleoli. In all cells investigated the nucleoli stain blue. In the less differentiated cells they generally appear darker blue than in the more mature cells, as e.g. in the bone marrow. Even in the fully developed leucocytes and lymphocytes blue nucleoli can be traced, though in some cells their presence is obscured by chromatin strands.

In addition to a nucleolus, a few extremely small dark blue stained particles, about 0.5 μ in diameter, are seen in some nuclei, particularly in tissue cultures. They are absent in most nuclei, but a minority contain either one or several particles. These granules are not associated with the nucleolus, but appear on the purple-red stained chromatin strands (Fig. 2, r).

B. *Dividing Cells.* In the course of cell division the chromosomes undergo striking changes in their staining reactions. At the beginning of cell division (prophase) they stain purple-red, but during meta- and anaphase they appear nearly black, due to a dark blue colour superimposed on the purple-red.

In telophase the chromonemal threads of the reconstituting daughter nuclei lose their blue staining component and stain purple-red as in prophase.

Prophase: Amongst the earliest changes which can be observed in cells about to divide is an increase in the thickness of the purple-red "chromatin strands" of the resting nucleus, whilst at the same time a few very small dark blue granules appear on the chromatin strands (Fig. 1, P_1). These granules are independent of the nucleolus, and their number and

---

Fig. 1–7. Chick embryo osteoblasts grown *in vitro* for 24 to 48 hours, fixed in methanol, stained whit May-Grunwald's and Giemsa's dyes. Cytoplasm and nucleoli: blue. Resting nuclei: purple-red.

Fig. 1 (×825). A 'premitotic' cell (p_1): in its nucleus appear a few, very small, blue particles on the thickened chromatin strands. An early prophase (p_2): most of its chromosomes stain purple-red, but a few stain black. A late prophase (p_3): most of its chromosomes stain as black as in the metaphase near the centre of the field.

Fig. 2 (×1100). Prophase (p): some chromosomes stain purple-red, others are black. Metaphase (m): all chromosomes stain black. Resting nucleus (r) which shows one or two minute, blue stained granules on the purplered chromatin.

Fig. 3 (×825). An early prophase (p). An early metaphase (m) with black chromosomes. A telophase (t) with some parts of the chromonemata black, others purple-red.

Fig. 4 (×825). Metaphase, in surface view, with black stained chromosomes.

Fig. 5 (×425). Metaphase (m) and anaphase (a) with black chromosomes. The cytoplasm between the two sets of anaphase chromosomes is dark blue. In the telophase (t) nuclei some chromonemata are stained purple-red, others are black. The remnants of the spindle area are almost colourless.

Fig. 6 (×825). An early anaphase: cytoplasm between the two groups of black stained chromosomes is of the same light blue colour as other parts of the cytoplasm.

Fig. 7 (×825). Three anaphases: each showing dark blue cytoplasm between the two groups of chromosomes. The latter are stained black. (Reproduced by permission of the J. of Physiol.)

Fig. 8 (×1100). Anaphase with intensely blue stained cytoplasm between the two groups of black stained chromosomes. Note round and elongated cytoplasmic inclusions (stained pink) outside the spindle area.

Fig. 9 (×825). After 30 minutes' digestion with ribonuclease: the cytoplasm has lost its basophilia and the nucleoli stain only pale grey. They are surrounded by purple-red chromatin strands. Prophase (p) chromosomes stain purple-red throughout. Note also the telophase (t) nucleus which shows only purple-red stained threads.

Fig. 11 (×825). After 30 minutes' digestion with ribonuclease: an early metaphase, its chromosomes stain purple-red; nucleoli, pale grey; chromatin, purple-red; cytoplasm, nearly colourless.

Fig. 12 (×1100). After 30 minutes' digestion with ribonuclease: metaphase chromosomes purple-red; chromatin of resting nucleus purple-red; cytoplasm and nucleoli nearly colourless.

Fig. 13 (×825). After 30 minutes' digestion with ribonuclease: anaphase chromosomes stain purple-red. The cytoplasmic area between chromosomes is quite colourless and empty, whereas the remainder of the cytoplasm is just visible due to cytoplasmic inclusions.

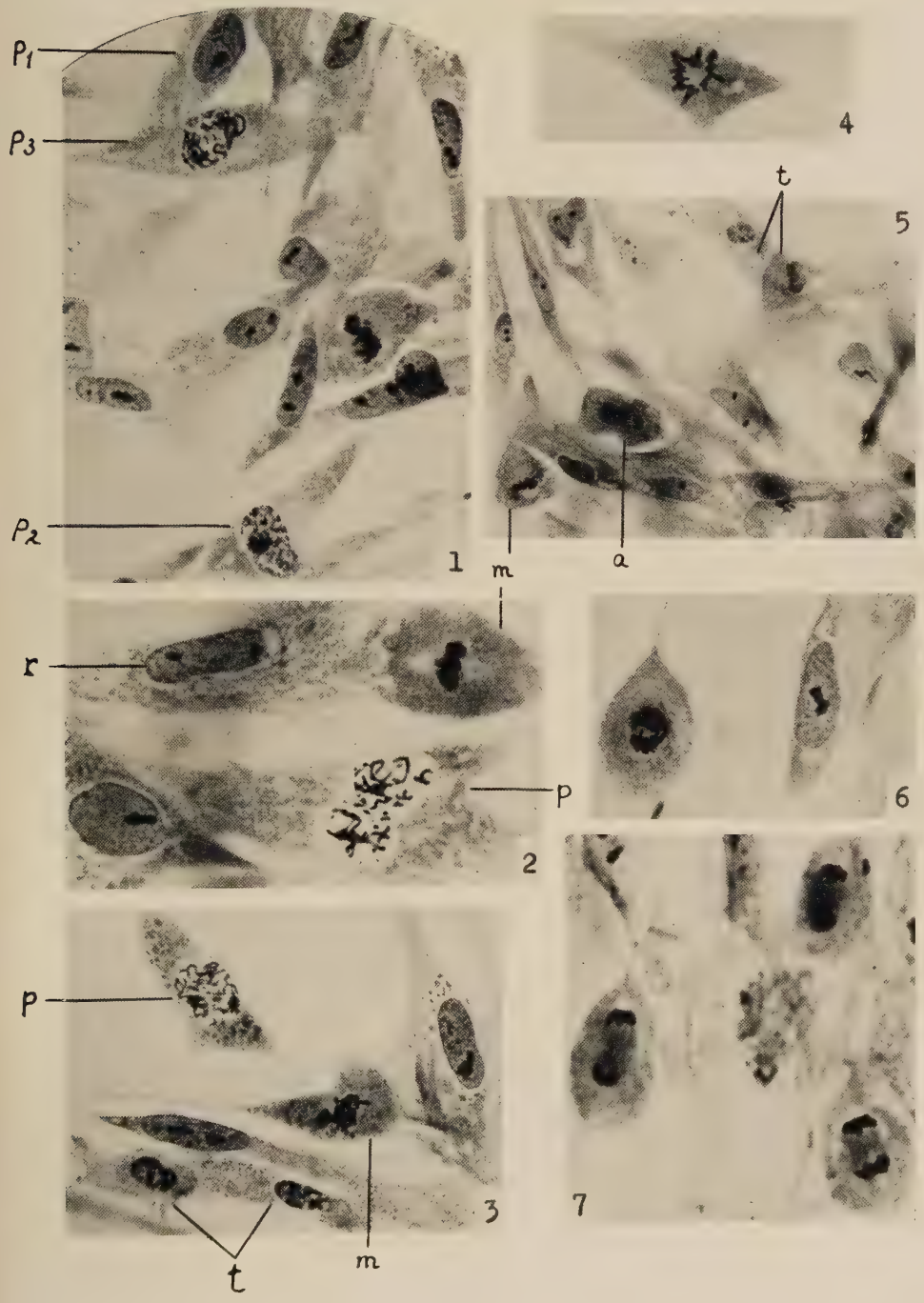
Fig. 14 (×825). After 45 minutes' digestion with deoxyribonuclease in the presence of hydroxylamine; resting nuclei look empty, except for the blue stained nucleoli. The cytoplasm is blue. In the prophase nucleus (p), many chromosomes are left colourless, but a few stain as dark blue as the nucleolus (at the end of the indication line).

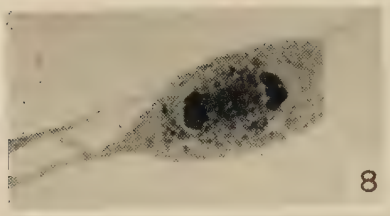
Fig. 15 (×825). After 45 minutes' digestion with deoxyribonuclease in the presence of hydroxylamine. Anaphase chromosomes are stained dark blue; cytoplasm blue, especially the area between the two groups of chromosomes. The resting nuclei appear empty; their nucleoli are dark blue.

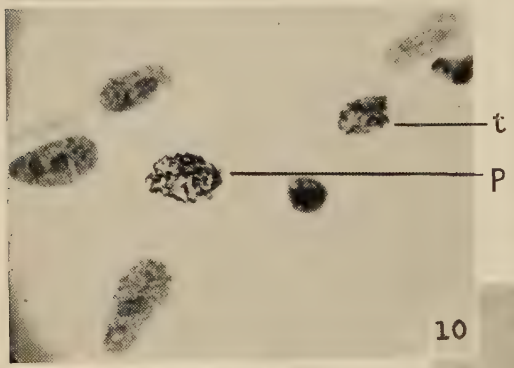
Errata in 3: 1

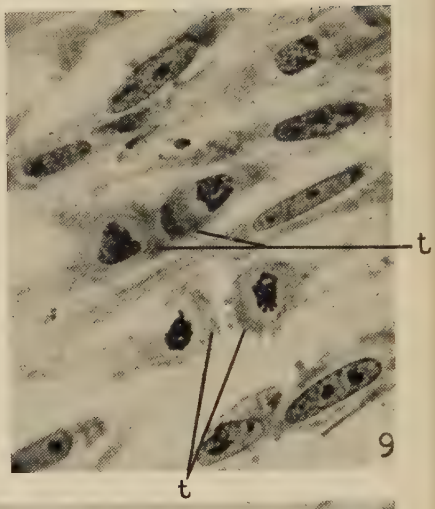
p. 168: Fig. 1–7 should read: *1–15.*
Fig. 9 should read: *Fig. 10.*

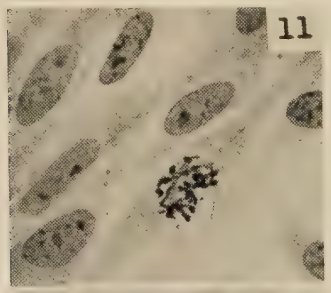
Text to: Fig. 9 (X 825). Two telephases (t); most of the chromonemata in their nuclei are stained purple-red. Indication lines point to the intensely blue stained region of the cytoplasm.

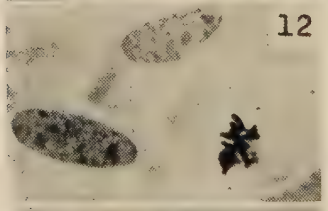

P1
P3
P2
1
4
t
5
m
a
r
P
2
6
P
3
7
t
m

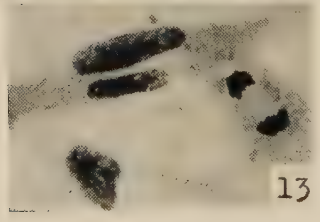

8

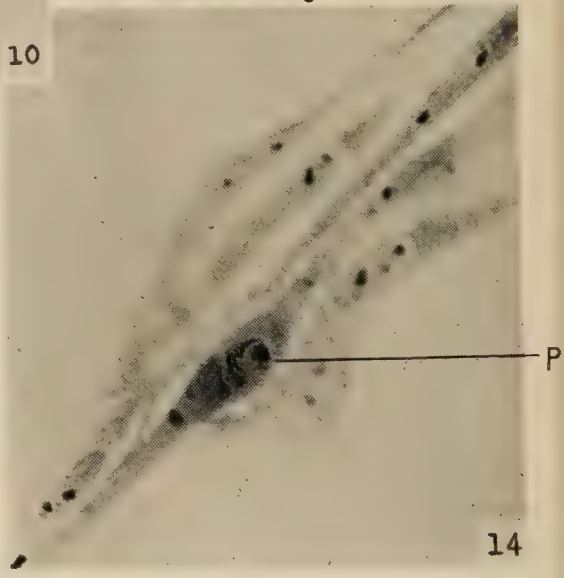

t
P
10

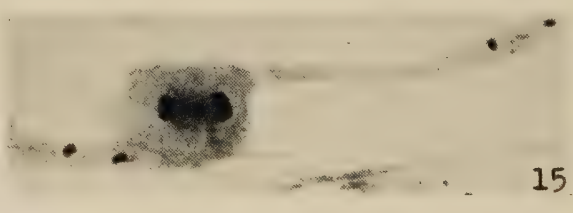

t
9
t


11


12


13


P
14


15

size increase concurrently with the increasing thickness of the chromosomal strands. Thus, in more advanced stages of the prophase some parts of the chromosomes may appear dark blue, almost black, while other parts of the same chromosome are still purple-red (Fig. 1, P_2 and P_3). Some of the dark blue material may now be found covering the chromosomal threads where they are attached to the nucleolus, but when the nuclear membrane breaks down, not all chromosomes stain black throughout their length.

Metaphase: When the chromosomes are gathered at the equator of the cell, in the fully developed metaphase, they stain almost black throughout (Fig. 4). This is due to a dark blue component superimposed on the purple-red. The amount of blue staining material on the chromosomes now appears considerably greater than in prophase. During this phase the cytoplasm does not appear to show any changes in the amount of blue stained material. The spindle fibres however appear as colourless strands (Fig. 2 and 5).

Anaphase: The anaphase chromosomes are stained dark blue almost black, like the metaphase chromosomes. During the very early anaphase the narrow cytoplasmic area between the two sets of chromosomes may be of the same colour as the remainder of the cytoplasm (Fig. 6). However in the more advanced anaphase, the cytoplasmic area between the two groups of chromosomes is stained darker blue than any other part of the cytoplasm (Fig. 5 and 7). The dark blue stained particles in this area are arranged in parallel strands, which are separated from each other by colourless spindle fibres. The spindle fibres near the cell poles also appear colourless, but the cytoplasm between the individual fibres may contain small blue staining particles.

The increased basophilia of the cytoplasm between the two groups of anaphase chromosomes cannot be due to mitochondria or other cytoplasmic inclusions as these do not generally occur in the area of the central spindle. This was observed by Fell and Hughes (13) on living cells with phase contrast microscopy (their Fig. G, Plate V) and was confirmed also in fixed preparations used in the present investigation (Fig. 8).

Telophase: When the nuclear membranes of the two daughter cell nuclei are formed, the chromosomal threads have lost some of the blue staining component. They now stain purple-red, although some parts retain their dark blue colour (Fig. 3 and 5 and 9). The dark blue staining substance which was between the two groups of chromosomes during the anaphase can still be traced in the cytoplasm outside the two daughter cell nuclei (Fig. 3 and 9).

When the daughter cell nuclei have been reconstituted one or two dark

TABLE I

Summary of results obtained with May-Grunwald and Giemsa's dyes.

Resting cells:	
Chromatin	purple-red
Nucleolus	blue
Nuclear membrane	colourless
Certain cytoplasmic material	blue
Mitosis:	
Prophase chromosomes	purple-red
Metaphase chromosomes	black (= purple-red + blue)
Anaphase chromosomes	black (= purple-red + blue)
Telophase chromosomes	purple-red
Spindle fibres	colourless
Cytoplasmic area between the two groups of anaphase chromosomes	blue

N. B. Late prophase and early telophase chromosomes show some blue material on the purple-red coloured threads.

blue staining nucleoli become visible and the chromatin threads stain purple-red. The results of staining with May-Grunwald and Giemsa dyes may be summarised as follows:—

II. *Isolated nucleoproteins and their components*

In the preceding section it was shown that the cytoplasm and the nucleolus stain blue with the May-Grunwald and Giemsa technique. Both these structures are known to contain ribonucleoprotein. On the other hand, the chromatin network, which is Feulgen positive, due to the deoxyribonucleoprotein, stains purple-red. Therefore the following *in vitro* tests with isolated nucleoproteins etc. were undertaken to demonstrate that the May-Grunwald and Giemsa method, as used in this work, stains ribonucleoproteins blue and deoxyribonucleoproteins purple-red. Various nucleoproteins, nucleic acids and proteins were suspended in a small volume of water and smeared on slides. After fixation in absolute methanol (10 minutes), the smears were stained with May-Grunwald and Giemsa stains according to the procedure described for tissues. The results obtained under these conditions are shown in Table II and indicate that deoxyribonucleoproteins stain purple-red, whereas the ribonucleoproteins stain dark blue.

p. 172: »may be summarised as follows» should read: *are summarised in Table I.*

TABLE II

Type	Colour
Deoxyribonucleoprotein from rye[1]	Purple-red
Deoxyribonucleoprotamine from herring sperm[2]	Purple-red
Deoxyribonucleohistone from calf thymus[2]	Purple-red
» » » » [3]	Purple-red
Deoxyribonucleic acid from H. pertussis[4]	Bright blue
Deoxyribonucleic acid from calf thymus[2]	Bright blue
Deoxyribonucleic acid from herring sperm[2]	Bright blue
Protamine (Eli Lilly & Co.)	Pink
Thymus histone[5]	Pink
Thymus deoxyribonucleic acid — protamine complex[6]	Purple-red
Herring sperm deoxyribonucleic acid — histone complex[6]	Purple-red
Herring sperm deoxyribonucleic acid — protamine complex[6]	Purple-red
Ribonucleoprotein from pancreas[7]	Dark blue
Ribonucleoprotein from the Gram complex of yeast[8]	Dark blue
Yeast ribonucleic acid (British Drug Houses)	Dark blue
Yeast ribonucleic acid — Gram protein complex[8]	Dark blue

The results also show that the colours obtained with the nucleoproteins are determined by both the protein and the nucleic acid component. The method does not distinguish between the two types of free nucleic acids. These however are unlikely to occur in the cells (See discussion p. 180). Therefore it may be assumed that the purple-red stained material in the cells is deoxyribonucleoprotein, and that the blue stained material in the cells is ribonucleoprotein. The latter occurs not only in the cytoplasm and the nucleolus, but also on the meta- and anaphase chromosomes. To confirm this assumption, cells were treated with specific nucleases, which attacked either the ribo- or the deoxyribonucleoprotein only. The results are described in the following section.

[1], [4] The preparations of these materials has been described by Laland, Overend and Webb (23), and by Overend, Stacey, Webb and Ungar (27) respectively.

[2] Isolated according to the method of Mirsky and Pollister (26).

[3] Isolated according to the method of Stern (34).

[5] Isolated by saturating a solution of thymus deoxyribonucleohistone with sodium chloride.

[6] Obtained by adding a solution of the nucleic acid in 0.14 *M* sodium chloride to a solution of the basic protein in 0.14 *M* sodium chloride. The resulting fibrous precipitate was collected by winding around a glass rod and was washed with 0.14 *M* sodium chloride.

[7] Isolated according to the method of Kerr and Seraidarian (21).

[8] Obtained according to the method of Henry, Stacey and Teece (18).

OBSERVATION ON CELLS STAINED BY OTHER METHODS

The effect of staining with methylgreen pyronin is well known: deoxyribonucleoproteins are stained green-blue, ribonucleoproteins red. It should be pointed out however, that with this method the chromosomes in all stages of mitosis stain greenish blue and that it does not reveal a very clear colour difference between the meta- and anaphase chromosomes on the one hand, and the prophase and telophase chromosomes on the other. It is of particular interest that when pyronin only is used, prophase and telophase chromosomes do not stain, but meta- and anaphase chromosomes stain bright red. Thus the assumption that during the latter stages of mitosis both types of nucleoprotein occur on the chromosomes receives further confirmation. Also, when the cells are stained with methylgreen pyronin the spindle fibres remain colourless, but the cytoplasmic area between the two sets of anaphase chromosomes stains a darker red colour than the surrounding cytoplasm. This indicates an increased amount of ribonucleoprotein. These findings are in complete agreement with the results obtained with the May-Grunwald and Giemsa technique.

In comparing the results of the May-Grunwald and Giemsa technique with those obtained by the Feulgen method, a striking parallelism is noted between the purple-red staining material and the Feulgen positive material. This applies to both, resting nuclei and chromosomes. The Feulgen method, of course, does not reveal the presence of material other than deoxyribonucleoprotein. Thus the chromosomes at meta- and anaphase are stained the same colour by the Feulgen method as they are at prophase and telophase. The results obtained by the above techniques are summarized in Table III.

Table III

Summary of staining nucleoproteins in cells by different methods.

Deoxyribonucleoproteins = DRNP
Ribonucleoproteins = RNP

	DRNP	RNP	Remarks
May-Grunwald-Giemsa	purple-red	blue	
Methylgreen-Pyronin	blue-green	red	RNP is not revealed if the same structure contains DRNP (e.g.meta- and anaphase chromosomes)
Pyronin alone	—	red	e.g. meta- and anaphase chromosomes
Feulgen	red	—	

THE EFFECT OF TREATMENT WITH NUCLEASES ON THE STAINING REACTIONS OF CELLS

A. *General principles of interpreting results obtained after enzyme treatment*

1) *Purity.* As already pointed out in the section dealing with the preparation of the enzymes, a very high degree of purity is essential. For instance, traces of a proteolytic enzyme present in the deoxyribonuclease liquified the cytoplasm and digested the cellular structures to such an extent that during 30–45 minutes' incubation most of the cells had either disappeared or were reduced to faintly staining 'ghosts'.

2) *Buffer.* Control material kept in buffer solutions alone, at 37° C for 30–45 minutes showed that the choice of a buffer was of some importance. In agreement with White's (40) and Sander's (31) observations, it was found that Michaelis' isotonic veronal-sodium acetate buffer of pH 6.8–7.2 preserves the basophilia of the cytoplasm, nucleoli, and meta- and anaphase chromosomes better than phosphate buffers. However it was noted that the use of buffer solutions which had been kept for some days resulted in a loss of ribonucleoprotein from the cells during 30 minutes at 37° C. This was shown to be due to the presence of a weak ribonuclease activity, which was associated with a slight contamination of the solution by bacteria or moulds.

An example of the ribonuclease activity which appeared in buffers as a result of contamination with microorganisms is shown by the following experiment. A filtrate from the contaminated isotonic veronal buffer was divided into two fractions, one of which was maintained at 100° C for 45 minutes to inactivate the enzyme. Each of these solutions (15 ml) was added to 0.2 per cent sodium ribonucleate (15 ml) and the mixture incubated at 37° C. At suitable intervals, aliquots of each solution were withdrawn and the extent of the hydrolysis of the nucleic acid determined by the increase in acid soluble phosphorus, according to the method of Davidson and Waymouth (11). No significant hydrolysis of the substrate occurred in the control (i.e. in the presence of the heated filtrate) during the period of the experiment (23 hours), whereas with the unheated filtrate, the hydrolysis of the ribonucleic acid amounted to 1.8, 4.9, 10.4, and 13.5 per cent after 1.5, 4.5, 16.0, and 23.0 hours respectively.

In view of these observations the buffer solutions used in the following experiments were either freshly prepared or kept under sterile conditions.

B. *The effect of treatment with nucleases*

Cells grown *in vitro* were used for the following digestion experiments. The cultures were fixed in absolute methanol for about 10 minutes, they were washed in 3 changes of water and then immersed in the enzyme solutions. The latter were prepared by diluting a concentrated solution of either ribo- or deoxyribonuclease with veronal-acetate buffer in the ratio of 1:4. The final concentration of the enzyme in these solutions was c. 0.075 per cent. After treatment with the enzyme solutions, the cultures were washed rapidly and transferred to absolute methanol, which was replaced once. They were then stained with May-Grunwald and Giemsa's solutions.

Ribonuclease. After 30–45 minutes' incubation at 37° C all blue staining material had been removed from the cells. Their cytoplasm was nearly colourless or faint pink, the nucleoli were pale grey, and meta- and anaphase chromosomes stained purple-red, as in prophase (Fig. 10–13).

Shorter periods of incubation (25 or 15 minutes) revealed a complete loss of cytoplasmic basophilia. The nucleoli were grey to light blue. Nearly all meta- and anaphase chromosomes were purple-red, but some showed traces of the blue component. After 10 minutes' incubation much the same picture was obtained, except that the cytoplasmic basophilia was not removed completely and stained light blue.

After 5 minutes incubation cytoplasm and nucleoli appeared pale blue. In some anaphases a concentration of blue staining material was present between the two groups of chromosomes, but in others it was digested away. Meta- and anaphase chromosomes showed a partial loss of the blue staining component, and varied in colour from purple-red to dark blue.

Control cultures kept for the same length of time in buffer (i.e. without enzyme) at 37° C, showed no appreciable loss of basophilia in the short term experiments. After 30–45 minutes a slight loss of basophilia was noted, but this in no way compared with the total loss of basophilic material which resulted from the action of ribonuclease.

In conclusion, it may be said that all basophil material was removed from the cells by ribonuclease within 30–45 minutes. The first basophil material to be attacked by the enzyme appeared to be the blue staining cytoplasmic component between the two groups of anaphase chromosomes and some of the blue staining substances on the meta- and anaphase chromosomes.

In agreement with these observations it was found that digestion with ribonuclease for 35 minutes also led to a complete removal of the pyronin

stainable material: cytoplasm and nucleoli failed to stain with pyronin and meta- and anaphase chromosomes no longer contained a pyronin-positive component. The methylgreen or Feulgen positive material in the resting nuclei and chromosomes was left intact by ribonuclease.

Deoxyribonuclease (in the presence of proteolytic inhibitors): After 30–50 minutes' digestion with deoxyribonuclease at 37° C no purple-red staining material was left in the resting nuclei. The nucleoli stained dark blue and stood out clearly in the nearly colourless nuclear substance (Fig. 14 and 15). Among the dividing cells the prophase chromosomes were reduced to pale grey strands and for this reason it was sometimes very difficult to identify prophases (Fig. 14). The chromosomal strands of telophases were reduced to nearly colourless threads with fine blue granules or strands while the meta- and anaphase chromosomes stained an intense blue. The cytoplasm retained most of its blue staining component and the darker blue colour of the cytoplasmic area between the anaphase chromosomes was still recognizable (Fig. 15).

After 25 minutes' action at 37° C the digestion with deoxyribonuclease was not complete. The resting nuclei contained a pale purple-red staining chromatin network; also prophase and telophase chromosomes were still visible as purple-red strands, though much fainter than the controls. Meta- and anaphase chromosomes appeared dark blue. The blue staining material of the cytoplasm and the nucleoli was not attacked by the enzyme.

In order to prove that the purple-red staining material removed by deoxyribonuclease was deoxyribonucleoprotein, cultures exposed to the enzyme at 37° C for 45 minutes were stained by the Feulgen method or with methylgreen pyronin. No Feulgen positive material was left in the cells. Resting nuclei and chromosomes, where these could be identified, did not show a trace of red or pink colouration. Furthermore after staining with methylgreen pyronin no green-blue coloured material could be found in resting nuclei or chromosomes. Prophase and telophase chromosomes appeared as faint grey strands. Meta- and anaphase chromosomes stained red with pyronin and so did the nucleoli in the empty-looking nuclei. The cytoplasm also stained red and showed the darker red colour in the area between the anaphase chromosomes.

C. *The specificity of the nucleases*

In a preceding paragraph it was shown, by chemical experiments *in vitro*, that ribonuclease and deoxyribonuclease, in the presence of proteolytic inhibitors, were specific for the corresponding nucleic acids (see (15)). In order to prove that this

specificity applies also to the action of these enzymes on the nucleoproteins or nucleic acids of the cells, the following experiments were undertaken.

The action of Lanthanum and Beryllium ions on the nucleoproteins. The nucleoproteins and/or nucleic acids were precipitated in the intact cells by immersing unfixed cultures in either lanthanum acetate (1 per cent) or, in later experiments, in isotonic beryllium sulphate ($BeSO_4 \cdot 4\ H_2O$, 2.6 per cent). The precipitating action of these metallic ions on nucleic acids and nucleoproteins was described by Caspersson, Hammarsten and Hammarsten (6), Webb (39) and Gilbert, Overend and Webb (16). The beryllium salt was used as it was considered that the smaller, divalent beryllium ion would penetrate into the unfixed cell more rapidly than the larger, trivalent lanthanum ion.

The compounds formed by the interaction of lanthanum or beryllium ions with thymus deoxyribonucleic acid and yeast ribonucleic acid were investigated *in vitro*. Neither the beryllium nor the lanthanum salts of either ribo- or deoxyribonucleic acid were digested to any measurable extent by their corresponding nucleases. The solubility of the lanthanum salts of thymus deoxyribonucleic acid was determined by Caspersson, Hammarsten and Hammarsten (6) as less than 0.001 per cent. The solubilities of the beryllium salts of the ribo- and deoxyribonucleic acids in water were found to be 0.0039 per cent and 0.00018 per cent respectively, as follows. A 1 per cent beryllium sulphate solution was added to 1 per cent solutions of the nucleic acids (100 ml) at 18° C until no further precipitation occurred. After 2 hours the resulting precipitates were collected by centrifuging, washed six times with water, and suspended in water (100 ml). The suspensions were shaken mechanically for 24 hours at 18° C and then centrifuged. Aliquots of each of the clear supernatants were removed, concentrated into a small volume in vacuo and analysed. Deoxyribonucleic acid was determined by the Dische (12) method and ribonucleic acid by the method of Euler and Hahn (12 a).

The following observations were made on the effects of lanthanum and beryllium salts on chick embryo fibroblasts and osteoblasts grown *in vitro*. The explants were placed, before fixation, in the lanthanum acetate or beryllium sulphate solution for 10 minutes or more. This resulted in precipitation of the nuclear and cytoplasmic nucleoproteins. After this the tissues were washed in saline, fixed in methyl alcohol and stained with May-Grunwald and Giemsa's dyes. The purple-red stained chromatin was arranged in fine granules and strands mainly on the inner surface of the nuclear membrane. The blue-stained cytoplasmic material had a more granular appearance. This treatment rendered the ribo- and deoxyribonucleoproteins considerably more resistant to the action of the corresponding nucleases. Thus, 30 minutes exposure to ribonuclease failed to remove the cytoplasmic basophilia between the anaphase chromosomes in cells of cultures treated with lanthanum or beryllium ions. Furthermore, the nucleoli and meta- and anaphase chromosomes in these cultures retained their basophilic components. In contrast, control cultures, which were not treated with these ions, when exposed to the enzyme lost the cytoplasmic basophilia between the anaphase chromosomes within 10 minutes and all basophilic material within 30 minutes.

Similarly, hardly any effect resulted from the action of deoxyribonuclease for 30–45 minutes on cells pre-treated with lanthanum or beryllium ions. Thus in these cells the purple-red chromatin of the resting nuclei, the prophase and telophase

chromosomes and the black colour of meta- and anaphase chromosomes did not differ appreciably from control cells which had not been exposed to the action of deoxyribonuclease.

D. *The effect of nuclease inhibitors on the digestion experiments*

Inhibition of deoxyribonuclease. Deoxyribonuclease in the presence of sodium arsenate (0.1 *M*) or zinc sulphate (0.10–0.01 *M*) failed to digest the purple-red stainable material in the interphase nuclei and in the chromosomes during 30 minutes at 37° C. These substances have been shown to inhibit the action of deoxyribonuclease *in vitro* (16). Also deoxyribonuclease which had been heated in boiling water for 30 minutes, showed no action on deoxyribonucleic acid *in vitro*, and was without effect on cell nuclei and chromosomes.

Inhibition of ribonuclease. The action of ribonuclease on cells was inhibited by zinc sulphate (0.1–0.01 *M*). No significant decrease of blue-stainable material was observed after 30 minutes at 37° C, compared with the controls kept in buffer solutions only. It had been shown by Zittle (44), that zinc prevents the action of the enzyme on isolated ribonucleic acids *in vitro*.

The above experiments show that the action of the nucleases was inhibited either by precipitation of the substrate with lanthanum or beryllium ions, or by the presence of specific enzyme inhibitors. It may therefore be concluded that in the digestion experiments each nuclease exerted a specific effect upon its substrate in the cells. The disappearance of blue or purple-red stainable material could not be attributed to some quite unspecific action, as for example, an increased solubility of the nucleoproteins in the enzyme buffer solutions.

DISCUSSION

With the material used in this investigation the May-Grunwald and Giemsa method is found to stain deoxyribonucleoprotein purple-red, and ribonucleoprotein blue. The specificity of the staining reaction is shown by the following observations.

1) The nucleolar and cytoplasmic structures which stain blue with the May-Grunwald and Giemsa technique stain red with pyronin. They are known to contain ribonucleoproteins and are Feulgen negative. After treatment with ribonuclease they cannot be stained by the above method.

The use of ribonuclease as a tool for demonstrating the presence of ribonucleoproteins in cells was described by Brachet (2, 3). His findings have been confirmed by Davidson and Weymouth (10), Wislocki and Dempsey (43), Stowell (35, 36), Sanders (31), Catcheside and Holmes (7), White (40), Davidson, Leslie and White (9) and others.

2) The purple-red staining chromonemata of the interphase nucleus (the

chromatin network of the resting nucleus) corresponds to the deoxyribonucleoprotein as shown by either the Feulgen or methylgreen method; the latter has been demonstrated to be specific for the deoxyribonucleic acid components of chromatin (30).

After digestion of the deoxyribonucleoprotein with deoxyribonuclease the chromatin can no longer be stained purple-red by the May-Grunwald and Giemsa technique, nor can it be demonstrated either by the Feulgen method, or with methylgreen.

Deoxyribonuclease as a tool for demonstrating the presence of deoxyribonucleoprotein in cells has been used by Sanders (31), Stowell (35, 36), Catcheside and Holmes (7), Brachet (4, 5), White (41), and others. Stein and Gerarde (33) have used this enzyme on tissue cultures. However in many cases the results have been disappointing, since only in some instances a selective loss of deoxyribonucleic acid was observed. One reason for this may be that deoxyribonuclease, as isolated from the pancreas according to McCarty's (24) method, is usually contaminated with a proteinase. The latter enzyme rapidly destroys deoxyribonuclease in aqueous solution; moreover, it also digests thin layers of tissues as used in these experiments. However in the presence of proteolytic inhibitors deoxyribonuclease has given satisfactory results.

Secondly, a possible interaction between the fixatives used (e.g. formalin or solutions containing certain metallic ions) and the deoxyribonucleoprotein may have rendered the latter no longer susceptible to the action of the enzyme (cf. Davidson (8)). Absolute methanol was found to be a good fixative for the material used in this investigation.

The specificity of the staining reactions, as described, derives further support from *in vitro* experiments with the isolated nucleoproteins (see Table II). Those of the ribose type stained bright blue, those of the deoxyribose type stained purple-red with the May-Grunwald and Giemsa technique.

Although this method differentiates the two types of nucleoproteins, it does not distinguish the free nucleic acids. Both the ribo- and the deoxyribonucleic acids stain blue (Table II). However, as far as is known, the nucleic acids appear to occur in the cell only in conjugation with a protein moiety. Moreover no evidence was obtained in the present investigation that digestion with deoxyribonuclease removed any blue staining material. This would occur if free deoxyribonucleic acid were present in the cell, since the free acid would stain blue by the May-Grunwald and Giemsa method.

The presence of deoxyribonucleoprotein in the chromosomes throughout all phases of mitosis is a well established fact. The occurrence of ribonucleo-

protein on meta- and anaphase chromosomes had previously been suggested by Brachet (3), Turchini (38), and Kaufmann, McDonald and Grey (20) in some plant and invertebrate material.

In the course of this investigation it was noted that when pyronin only was used, meta- and anaphase chromosomes stained bright red as for ribonucleoproteins. Prophase and telophase chromosomes were left colourless with the exception of small discrete areas or parts of the chromosomes. Thus the present investigation fully confirms previous findings. Owing to the intense blue colour given by the ribonucleoprotein with the May-Grunwald and Giemsa stain it was possible to extend these observations to the first appearance and the removal of ribonucleoproteins from the chromosomes during the mitotic sequence.

There are two points of interest concerning the first appearance of ribonucleoprotein on the chromosomes. Firstly, ribonucleoprotein appears on the chromosome during prophase, especially the latter part of prophase, before the nuclear membrane has gone and while the nucleolus is still present and of normal size. No decrease in cytoplasmic nucleoprotein has been observed during any phase of mitosis in our material. Secondly, ribonucleoprotein appears on some prophase chromosomes in direct continuity with the nucleolus, but on others frequently as discrete particles anywhere along the chromosomal threads. It may therefore be concluded that there is no evidence that the entire ribonucleoprotein of the chromosome is derived from the nucleolus.

During anaphase movement ribonucleoprotein is found in the cytoplasmic area between the two groups in a concentration higher than in any other part of the cytoplasm. This increased amount of ribonucleoprotein can be demonstrated very clearly by the May-Grunwald and Giemsa method, when it is stained dark blue. It can also be shown with pyronin as a slightly darker red stained area between the two groups of anaphase chromosomes, and is observed not only in cells fixed in methanol, but also in some cells after fixation in Susa, Bouin's or Zenker's fluid.

For the following reasons it is considered unlikely that this increased basophilia results from the adsorption of cytoplasmic ribonucleoprotein on the spindle fibres:

1) The area of the spindle fibres in metaphase does not show an increased basophilia; 2) In some, but not all very early anaphases, when the two groups of chromosomes have just separated from the metaphase plate, no increase in basophilia is observed in the narrow cytoplasmic area between the two groups of chromosomes. This area still shows the same colour as

the surrounding cytoplasm; 3) In all anaphases the spindle fibres themselves remain colourless threads passing through this area and 4) The cytoplasm adjacent to the spindle fibres does not show any decrease in basophilia as it might be expected if they had adsorbed ribonucleoprotein from the surrounding cytoplasm.

The possibility, therefore, suggests itself that ribonucleoprotein is transferred from the chromosomes into the cytoplasm during the anaphase of cell division. This process may have considerable biological significance.

SUMMARY

The two types of nucleoproteins were studied in resting cells and during mitosis by the May-Grunwald and Giemsa method, following fixation in absolute methanol. Normal cells, cells grown *in vitro* and leukaemic cells of a transmissable acute leukaemia in a hybrid FAK_1 mouse strain were used for this investigation. It was found that deoxyribonucleoproteins stained purple-red, and ribonucleoproteins blue. This was also shown by staining the isolated nucleoproteins.

Deoxyribonucleoproteins occur in the chromosomes of the resting nucleus and in the chromosomes throughout all phases of mitosis. Ribonucleoproteins were found in the cytoplasm of all undifferentiated cells, in the nucleoli of all cells investigated and in the chromosomes from the end of prophase, through meta- and anaphase, up to early telophase. During anaphase ribonucleoprotein appears to be shed from the chromosomes into the cytoplasm between the two groups of anaphase chromosomes.

Ribonuclease digests away all ribonucleoprotein. Deoxyribonuclease, in the presence of proteolytic inhibitors, removes from the cells deoxyribonucleoproteins. Neither enzyme acted when specific inhibitors were added. When the cells were treated with either lanthanum or beryllium ions, the resulting nucleoprotein complexes were not attacked by their respective nucleases in the times sufficient for the digestion of the nucleoproteins in untreated cells.

The authors gratefully acknowledge the many helpful discussions throughout this work, with Dr. H. B. Fell, Director of the Strangeways' Research Laboratory. The authors are indebted to Miss M. Fell and Miss J. Rawlinson for their help in setting up tissue cultures and to Miss M. Fell for help in preparing the manuscript for publication. The expenses of this investigation were defrayed by the Medical Research Council and the Nuffield Foundation.

REFERENCES

1. Brachet, J., *Compt. Rend. Soc. Biol.*, **133**, 88 (1940).
2. —— *Enzymologia*, **10**, 87 (1941).
3. —— *Arch. Biol.*, **53**, 207 (1942).
4. —— Over Proteinen. Biochemische Gehoulden Vlaamsche Chemische Vereenigung, Antwerp, 1943.
5. —— Nucleic Acids. Symposia Soc. Exptl. Biol., No. 1, Cambridge University Press, p. 207, 1947.
6. Caspersson, T., Hammarsten, E., and Hammarsten, H., *Trans. Faraday Soc.*, **31**, 367 (1935).
7. Catcheside, D. G., and Holmes, B., Nucleic Acids. Symposia Soc. Exptl. Biol. No. 1, Cambridge University Press, p. 225, 1947.
8. Davidson, J. N., *Cold Spring Harbor Symposia Quant. Biol.*, **12**, 50 (1947).
9. Davidson, J. N., Leslie, I., and White, J. C., *J. Path. Bact.*, **60**, 1 (1948).
10. Davidson, J. N., and Waymouth, C., *Proc. Roy. Soc. Edinb.*, **62** (B), 96 (1944).
11. —— *Biochem. J.*, **38**, 375 (1944).
12. Dische, Z., *Mikrochimie*, **8**, 4 (1930).

12 a. Von Euler, H., and Hahn, L., *Svensk Kem. Tid.*, **58**, 251 (1946).

13. Fell, H. B., and Hughes, A. F., *Quart. J. Microscop. Sci.*, **90**, 355 (1949).
14. Fischer, F. G., Boettger, I., and Lehmann-Echternacht, H., *Z. Physiol. Chem.*, **271** 246 (1941).
15. Gilbert, L. M., Overend, W. G., and Webb, M., *Exptl. Cell Res.*, **2**, 138 (1951).
16. —— *ibid.*, **2**, 349 (1951).
17. Grob, D., *J. Gen. Physiol.*, **29**, 219 (1946).
18. Henry, H., Stacey, M., and Teece, E. G., *Nature*, **156**, 720 (1945).

18 a. Jacobson, W., Biology of Mitosis. 13th Conference on Problems of Ageing, Josiah Macy Jr. Foundation, New York, 1951.

19. Jacobson, W., and Webb, M., *J. Physiol.*, **112**, 2 P (1951).
20. Kaufmann, B. P., McDonald, M., and Grey, H., *Nature*, **162**, 814 (1948).
21. Kerr, S. E., and Seraidarian, K., *J. Biol. Chem.*, **180**, 1203 (1949).
22. Kunitz, M., *J. Gen. Physiol.*, **24**, 15 (1940).
23. Laland, S., Overend, W. G., and Webb, M., *Acta Chem. Scand.*, **4**, 885 (1950).
24. McCarty, M., *J. Gen. Physiol.*, **29**, 123 (1946).
25. McDonald, M. R., *ibid.*, **32**, 39 (1948).
26. Mirsky, A. E., and Pollister, A. W., *ibid.*, **30**, 117 (1946).
27. Overend, W. G., Stacey, M., Webb, M., and Ungar, J., *J. Gen. Microbiol.*, **5**, 268 (1951).
28. Overend, W. G., and Webb, M., *J. Chem. Soc.* p. 2746 (1950).
29. Peters, R. A., Wakelin, R. W., and Cecil, R., *Biochem. J.*, **43**, 45 (1948).
30. Pollister, A. W., and Leuchtenberger, C., *Proc. Natl. Acad. Sci.*, **35**, 111 (1949).
31. Sanders, F. K., *Quart. J. Microscop. Sci.*, **87**, 203 (1946).
32. Schales, O., Suthan, A. M., Roux, R. M., Lloyd, E., and Schales, S. S., *Arch. Biochem.*, **19**, 119 (1948).
33. Stein, R. J., and Gerarde, H. W., *Science*, **111**, 256 (1950).
34. Stern, K. G., *Exptl. Cell Res. Suppl.* **1**, p. 97 (1949).
35. Stowell, R. E., *Cancer Res.*, **6**, 426 (1946).
36. —— *Stain Technol.*, **21**, 137 (1946).
37. Thorell, B., *Acta Med. Scand.*, **200**, Suppl. (1947).
38. Turchini, J., *Exptl. Cell Res. Suppl.* **1**, p. 105 (1949).
39. Webb, M., *J. Gen. Microbiol.*, **5**, 580 (1951).
40. White, J. C., *J. Path. Bact.*, **59**, 223 (1947).
41. —— *Biochem. J.*, **46**, xxiv (1950).
42. Wintrobe, M. M., Clinical Hematology, 2nd ed., Henry Kimpton, London, 276, 1946.
43. Wislocki, G. B., and Dempsey, E. W., *Anat. Rec.*, **96**, 249 (1946).
44. Zittle, C. A., *J. Biol. Chem.*, **163**, 111 (1946).

VITAMIN C MICROSCOPIC DEMONSTRATION AND GOLGI APPARATUS

JULIO MARÍA SOSA

Neurological Institute and Research Institute for Biological Sciences, Montevideo, Uruguay

Received August 15, 1951

In previous publications (9, 10) the problem of the specificity of Giroud-Leblond's silver reduction method (3, 4) was very briefly discussed, and some results of experiments on the subject were advanced.

The starting point arose from the doubt that sodium thiosulphate could be a disturbing factor which, by direct action upon some silver nitrate remnant (remaining in spite of the washing), would produce silver salt precipitates, having no bearing on the reduction of silver nitrate promoted by the ascorbic acid.

In fact, if a silver nitrate solution (even acetified in the usual manner) is kept in a test-tube, and a sodium thiosulphate solution is slowly dropped in it, some heavy white flocks appear, which quickly turn successively yellow, reddish-brown, and finally black, coming down to the bottom of the test-tube.

This fact has been well known to chemists (1, p. 632). Yet, a similar phenomenon had already been observed and described by Herschel (1819) (7, p. 536) when letting the silver nitrate react upon potassium thiosulphate.

Now, if an excess of sodium thiosulphate is suddenly dropped on a weak silver nitrate solution, a slight opalescence appears (silver thiosulphate), which immediately disappears by transformation in thiosulphate of silver and sodium. However, whether the silver nitrate solution is relatively strong or the sodium thiosulphate solution is slowly dropped on the silver solution, the white precipitate of silver thiosulphate takes the above mentioned colour variations, with the final appearance of a black precipitate (silver sulphide), which is no longer soluble in an excess of sodium thiosulphate.

RESULTS

Direct action of the thiosulphate upon silver nitrate. In order to determine exactly the degree of intervention of the factors *concentration* and *time*, test-tube experiments, summarized in the four series of fig. 1, have been carried out.

CORRECTION

SOSA, JULIO MARÍA. Vitamin C microscopic demonstration and Golgi apparatus. *Exptl. Cell Res.*, **3**, 184 (1952).

— In Table I, the mark *x* appearing in Series III, No. 7, under the vertical column *L* (limpid), should be rubbed out and put under the vertical column *T* (turbid).

— At the end of the explanation of Fig. 1, where it says *limpid.*, should say *limpid*; *ut. 5, up to 5 cc.*

— In Table II, the heading of the sixth vertical column says $\overset{10\ \%}{NO_2SO_3}$ when it should say $\overset{10\ \%}{Na_2SO_3}$

As a result of the experiments of series I, II and III, when sodium thiosulphate is suddenly poured, it becomes clear that: 1) a real great excess of sodium thiosulphate is required to dissolve the silver thiosulphate precipitate, avoiding its decomposition with final production of silver sulphide; 2) the stronger the silver nitrate solution is, the greater must be the excess of sodium thiosulphate; 3) when silver nitrate solution is strongly concentrated (10 per cent), even 40 volumes of 10 per cent sodium thiosulphate solution are not sufficient to impede the turbid appearance of the final liquid, which means a suspension of a relatively great amount of fine granules or, in other words, the production of some abundant *microscopic precipitate.*

Although the experiments of series I, II and III undoubtedly enable us to study some aspects of the problem of the action of sodium thiosulphate upon silver nitrate, they are not quite suitable to be compared with the intimate phenomena of the silver technique for the cytochemical detection of vitamin C. In fact, the quicker the phenomena might develop in the cytochemical technique, it is not to be expected that the action of the sodium thiosulphate upon silver nitrate may develop inside tissues as quickly as when the thiosulphate solution is suddenly poured on the silver nitrate solution in the test-tube.

The experiments of series IV were performed with the aim of approaching the problem of the chemical process inside cells and tissues. For that purpose, the excess of 10 per cent sodium thiosulphate solution was poured, drop by drop, on the different silver solutions, used at equal conditions of volume and concentration as in the three previous series.

The decomposition of the silver thiosulphate, ending in the black silver sulphide precipitate, appears like a spontaneous phenomenon, quite independent of any actinic influence of the light, for the same results are obtainable in the dark room, whether the experiments are performed under a red light or in the complete dark. In the latter case, the black precipitate has been found when switching the light on to see the result in the test-tube.

Still, assays have been also repeated with ascorbic acid. When an excess of silver nitrate is poured on an ascorbic acid solution in a test-tube, a net precipitate of reduced silver is, of course, obtained. Now, if a sodium thiosulphate solution is added, drop by drop, in the same test-tube, it reacts upon the silver nitrate excess, producing a white precipitate, which finally becomes black like in the cases shown in figure 1. This silver sulphide precipitates down to the bottom of the test-tube and is added to the precipitate of silver reduced by the ascorbic acid.

By considering all of these results, it appears clear that *time* acts as an important factor, together with the factor *concentration*, in determining either the production or the prevention of silver sulphide precipitate.

Se.	No.	$AgNO_3$					CH_3 | 1 % CO.OH in 10 % $AgNO_3$	10 % $AgNO_3$ in CH_3 | 10 % CO.OH	10 % $Na_2S_2O_3$		Precipitate				Final liq.		
		0.5 %	1 %	2 %	5 %	10 %			Q	su. sl.	G	M	S	N	T	O	L
I	1	0.5							5	×				×			×
	2		0.5						»	×				×			×
	3			0.5					»	×				×			×
	4				0.5				»	×				×			×
	5					0.5			»	×		×					×
	6						0.5		»	×		×					×
	7							0.5	»	×		×			×		
II	1	0.5							10	×				×			×
	2		0.5						»	×				×			×
	3			0.5					»	×				×			×
	4				0.5				»	×				×			×
	5					0.5			»	×				×			×
	6						0.5		»	×				×			×
	7							0.5	»	×			×		×		
III	1	0.5							20	×				×			×
	2		0.5						»	×				×			×
	3			0.5					»	×				×			×
	4				0.5				»	×				×			×
	5					0.5			»	×				×			×
	6						0.5		»	×				×			×
	7							0.5	»	×				×			×
IV	1	0.5							ut. 5	×				×			×
	2		0.5						»	×				×			×
	3			0.5					»	×			×			×	
	4				0.5				»	×		×			×		
	5					0.5			»	×	×				×		
	6						0.5		»	×	×				×		
	7							0.5	»	×	×				×		

Fig. 1. Table representing the four series of experiments on the action of sodium thiosulphate upon silver nitrate when concentration and time factors are varied. The quantitities of the solutions used are expressed in cc. Se., series; Q, quantities; su., suddently poured; sl., slowly poured; G, great; M, medium; S, small; N, nil; T, turbid; O, opalescent; L, limpid.

It also becomes clear that there is a serious basis to assume that the sodium thiosulphate may, during the process of Giroud-Leblond method, react upon the free silver nitrate excess (not reduced by the vitamin) incidentally remain-

Se.	No.	10% $AgNO_3$	CH_3 | 1% CO.OH in 10% $AgNO_3$	10% $AgNO_3$ in CH_3 | 10% CO.OH	10% NO_2SO_3		10% $Na_2S_2O_3$		Precipitate				Final liq.		
					Q	W	Q	sl.	G	M	S	N	T	O	L
	1	0.5			0.5	×	5	×	×				×		
	2	»			1	×	»	×	×				×		
	3	»			2	×	»	×		×				×	
	4	»			3	×	»	×		×				×	
	5	»			4	×	»	×			×			×	
V	6	»			5	×	»	×				×		×	
	7	»			6	×	»	×				×			×
	8	»			7	×	»	×				×			×
	9	»			8	×	»	×				×			×
	10	»			9	×	»	×				×			×
	11	»			10	×	»	×				×			×
	1		0.5		0.5	×	»	×	×				×		
	2		»		1	×	»	×	×				×		
	3		»		2	×	»	×		×				×	
	4		»		3	×	»	×			×			×	
	5		»		4	×	»	×				×		×	
VI	6		»		5	×	»	×				×		×	
	7		»		6	×	»	×				×			×
	8		»		7	×	»	×				×			×
	9		»		8	×	»	×				×			×
	10		»		9	×	»	×				×			×
	11		»		10	×	»	×				×			×
	1			0.5	0.5	×	»	×	×				×		
	2			»	1	×	»	×	×				×		
	3			»	2	×	»	×		×			×		
	4			»	3	×	»	×			×		×		
	5			»	4	×	»	×				×		×	
VII	6			»	5	×	»	×				×		×	
	7			»	6	×	»	×				×			×
	8			»	7	×	»	×				×			×
	9			»	8	×	»	×				×			×
	10			»	9	×	»	×				×			×
	11			»	10	×	»	×				×			×

Fig. 2. Table representing the three series of experiments on the results of introducing sodium sulphite in the reaction of sodium thiosulphate upon silver nitrate. W, white precipitate. Other lettering like in fig. 1.

ing inside cells and tissues (in spite of the wash), producing granules of silver sulphide, impossible to distinguish from the granules due to the reducing action of the vitamin.

All of this does not mean that the use of sodium thiosulphate should be proscribed from the silver technique for the vitamin detection. In fact, it is well known that sodium thiosulphate (the photographers' "hypo") is ordinarily used as a photographic "fixer," because of its property to dissolve halides. This property makes the use of sodium thiosulphate necessary in the technique we are dealing with, to take out the silver chloride formed by action of silver nitrate upon cellular chlorides. If this precaution were not taken, the silver chloride, insoluble in water, which might only incompletely be removed by washing, would give rise to precipitate granules of reduced silver by the reducing activity of the light, so as to increase the microscopic picture given by the genuine silver reduced granules produced by vitamin action. The result would therefore be the obtention of an erroneous microscopical picture, suggesting an amount of vitamin C greater than the real one.

However, if the convenience of taking the silver chloride out, makes necessary the use of sodium thiosulphate after the action of the acetified silver nitrate solution, the foregoing considerations about the *in vitro* experiments suggest how much is needed to find out a procedure to avoid the disturbing action of sodium thiosulphate when it operates slowly upon the silver nitrate.

The introduction of sodium sulphite prior to the action of sodium thiosulphate. The black precipitate of silver sulphide produced by action of sodium thiosulphate upon silver nitrate in the test-tube, may be avoided by pouring a sodium sulphite solution prior to the thiosulphate. In fact, the addition of sodium sulphite to a silver nitrate solution produces a white precipitate of silver sulphite, which may immediately be dissolved by an excess of thiosulphate. This property has been known for long, because Mellor (7, p. 280) quotes that Herschel found (as early as 1819–20) that silver sulphite is easily soluble in a solution of alkaline thiosulphate.

For the purpose of finding out, as accurately as possible, under which conditions this phenomenon occurs, the three series of experiments shown in fig. 2 were undertaken. The observation of table representing series V, VI and VII, shows that: a) When the whole free silver nitrate is precipitated under the form of silver sulphite, the latter is completely dissolved by subsequent drop by drop addition of sodium thiosulphate, without any resulting silver sulphide precipitate; b) From eight to ten volumes of 10 per cent sodium sulphite are needed to avoid the formation of macroscopic precipitate, perfectly visible in the test-tube; c) To have a final clean liquid and, therefore, not even a microscopic precipitate, twelve volumes of 10 per cent sodium sulphite are required to react upon one single volume of 10 per

cent silver nitrate, prior to the slow dropping of 10 per cent sodium thiosulphate.

It still must be added that some control experiments with ascorbic acid have been carried out as follows: 1) by allowing an excess of silver nitrate to react upon a weak ascorbic acid solution, by means of which the reduction of some silver nitrate is obtained, giving origin to metallic silver precipitate (small brilliant silver flakes); 2) by adding an excess of sodium sulphite solution, with production of the silver sulphite precipitate; and, 3) by finally pouring, drop by drop, the thiosulphate solution whereby the white precipitate disappears, while the metallic silver precipitate remains without any black precipitate of silver sulphide.

Application of the chemical data to the improvement of the cytochemical technique. The *in vitro* experiments, then give a basis in support of the modification introduced to the cytochemical silver technique (9). It consists in the use of 10 per cent sodium sulphite solution after the action of the acetified silver nitrate (pH 4), and prior to the 10 per cent thiosulphate stage.

The technique is accounted for by the treatment, in the dark, at room temperature, of small pieces of tissues, not exceeding one or two millimetres thickness, according to the following stages:

I.—Immersion for one hour in: 10 per cent silver nitrate aqueous solution 100 cc, plus glacial acetic acid 1 cc. II.—Immersion for one hour in about 15 to 20 volumes (in relation to the whole volume of the pieces) of 10 per cent sodium sulphite. III.—Immersion for one hour in about 10 volumes of 10 per cent sodium thiosulphate. IV.—Wash in distilled water, dehydrate in the alcohols, and paraffin embedding. V.—Sections of 5–7 μ are counterstained with any nuclear red stain, like Safranin, and some pinky cytoplasmic stain, like eosin. Eosin is used instead of methylene blue, for better contrast. In this way, the black granules of silver precipitate may be easily seen in the cytoplasm, against the pinky background. Nuclear staining with Carazzi haematoxylin plus eosin cytoplasm staining give also excellent results.

DISCUSSION

The constant appearance of spherical granules, irregularly distributed inside the cytoplasm with no special topographic relation to particular organules, indicates that the Golgi-like pictures sometimes found in corpora lutea and adrenals of guinea pig and rat by using the classical method, only represent a *silver impregnation* of the Golgi apparatus. In fact, it follows as probable from a comparative study of the microscopic pictures given by the classical method and those obtained by the present technique that the granules shown by this last technique correspond to vitamin C, whereas some of the pictures given by the classical technique represent technical artefacts. The Golgi-like pictures and some other structure occasionally stained (connective tissue fibres, nuclear and cellular membranes, etc.), that may sporadically

be shown by the classical technique, are never seen when the action of sodium sulphite is introduced according to the above description. The artefacts may be the result of the following process: 1) adsorption of non-reduced silver nitrate by the above mentioned elements, particularly by the Golgi apparatus; 2) production of silver thiosulphate, because of the sodium thiosulphate action; 3) decomposition of the silver thiosulphate, with subsequent formation of silver sulphide precipitate, which results in more or less complete silver impregnation pictures of the adsorbent structures. These pictures may then be seen together with the true vitamin C granules, and complicate the cytochemical picture.

Moreover, some recent papers (2, 8) have brought some evidence against a complete specificity of the classical method.

It is claimed that pictures suggesting the localization of vitamin C either inside Golgi apparatus or on the mitochondria would mean an artefact due to a relative slowness of the fixing process. This has, however, been contested by Hoch-Ligeti and Bourne (6) in their paper on the changes in the concentration and histological distribution of the ascorbic acid in the ovary, adrenal and liver of the rat during estrual cycle.

The accumulation of the granules in a juxtanuclear zone must not necessarily mean their localization inside the Golgi apparatus or even inside the Golgi zone. In fact, it is well known that the Golgi apparatus has not necessarily a juxtanuclear situation in the adult ovarian, suprarenal and hepatic cells. Secondly, only when a technique capable of demonstrating simultaneously the microscopic picture of vitamin C granules and wholly stained Golgi apparatus (as completely as when using the classical either silver or osmic methods) would give an objective basis for accepting that a storage of vitamin C occurs inside or on the Golgi apparatus. Furthermore, even in cells with the Golgi apparatus constantly localized in a particular juxtanuclear pole, as for example in some exocrine cells, the accumulation of vitamin C granules in this zone does not necessarily mean that they are localized inside the Golgi apparatus. They may be accumulated in the cytoplasm surrounding the apparatus or amongst certain constituent elements of this latter.

While there is no method for simultaneously and completely revealing the Golgi apparatus and the vitamin C granules, Hirsch's theory (5) of the active part taken by the Golgi apparatus in the vitamin C storage in cell, should not be accepted either. Meanwhile, Sluiter's statement (8) that "it seems doubtful that a granular storage of vitamin C in the Golgi apparatus should occur" seems quite justified.

SUMMARY

Sodium thiosulphate may operate directly upon the silver nitrate adsorbed by various cellular and tissular structures, giving rise to a black precipitate of silver sulphide. This precipitate would give rise to a technical artefact, appearing as something added to the genuine picture given by the direct reduction of silver nitrate by the vitamin.

The application of a cytochemical method, involving adequate use of sodium sulphite after the acid silver nitrate and prior to the thiosulphate, prevents the silver sulphide precipitate, and shows: a) that vitamin C appears only in the form of granules, irregularly distributed in the cytoplasm; b) that vitamin C storage inside the Golgi apparatus or mitochondria never occurs; c) that the Golgi-like pictures that may occasionally be observed when using the classical silver method, do most probably represent silver impregnation pictures of the Golgi apparatus. This must not be accepted as an equivalent of the silver nitrate reduction by vitamin C stored in the Golgi apparatus.

REFERENCES

1. Calvet, E., *Quimica General*, **3**, 632 (2nd édit., Salvat) (1944).
2. Deane, H. W., and Morse, A., *Anat. Rec.*, **100**, 127 (1948).
3. Giroud, A., *Protoplasma Monographien*, **16**, 1 (1938).
4. Giroud, A., and Leblond, C. P., *Arch. Anat. Microscop.*, **30**, 105 (1934).
5. Hirsch, G. C., *Protoplasma Monographien*, **18**, 1 (1939).
6. Hoch-Ligeti, C., and Bourne, G. H., *Brit. J. Exptl Path.*, **29**, 400 (1948).
7. Mellor, J W., A comprehensive treatise of inorganic and theoretical chemistry, **10**, Longmans, Green & Co., London 1930.
8. Sluiter, J. W., *Proc. Koninkl. Nederland. Akad. Wetenschap.*, **51**, (1948).
9. Sosa, J. M., *Exptl. Cell Res., Suppl. I*, 402 (1949).
10. —— *Rev. Sudamericana Morphol.*, **6**, 115 (1948).

DETERMINATION OF THE RATE OF RENEWAL FROM THE RATE OF DISAPPEARANCE OF LABELLED MOLECULES

GEORGE HEVESY

Institute for Research in Organic Chemistry, University of Stockholm, Sweden

Received July 15, 1951

The rate of renewal of a type of molecules is usually calculated from the rate of incorporation of the labelled atoms of the pertinent precursor in the molecules considered. If we wish, for example, to know the percentage of desoxyribonucleic acid molecules of a rat tumour, which are formed in the course of two hours, we administer labelled sodium phosphate to a rat and two hours later we compare the specific activities of the desoxyribonucleic acid P and inorganic P extracted from the tumour. If the ratio of these specific activities is found to be 0.02, and the specific activity of the inorganic P remained constant during the experiment, we conclude that in the tumour 2 per cent of the desoxyribonucleic acid molecules are present which were formed during the experiment or, more correctly, that at least 2 per cent of these molecules were formed in the course of two hours. If a stock of desoxypentose phosphate is present in the tumour, it is possibly desoxypentose phosphate which is incorporated in the desoxyribonucleic acid molecules, and this compound reaches a comparatively slow exchange equilibrium with the labelled inorganic P, some of the desoxyribonucleic acid molecules are formed from inactive desoxypentose phosphate during the experiment lasting two hours. Thus, some of the newly formed desoxyribonucleic acid molecules are not labelled and the calculation of the rate of renewal of desoxyribonucleic acid molecules from specific activity data, obtained from the specific activities of inorganic P and desoxyribonucleic acid P, leads to a too low value of the renewal rate.

If we protract the experiment, the specific activity of the inorganic P of the tumour decreases more and more and this decrease is followed by a decrease in the specific activity of desoxyribonucleic acid P. We can also calculate the rate of renewal by comparing the specific activities of desoxyribonucleic acid P and inorganic P in this declining activity phase of the experiment, but again we encounter the difficulty that we do not know, whether inorganic

P is the pertinent precursor of desoxyribonucleic acid or not.[1] The decrease in the specific activity of desoxyribonucleic acid P in this late phase of the experiment is a result of the disappearance of strongly active molecules and the formation of less active ones.

The rate of loss of P^{32} by desoxyribonucleic acid molecules in the late phase of the experiment is independent of the precursor problem, but parallel with a loss of P^{32} by strongly active "old" desoxyribonucleic acid molecules the formation of less active "new" molceules takes place and the rate of incorporation of P^{32} in these molecules is partly determined by the specific activity of the pertinent precursor. Thus by replacing the calculation of the renewal rate from increasing values of the specific activities of desoxyribonucleic acid with time by a calculation based on decreasing specific activity values with time, we cannot eliminate the difficulty arising from the lack of knowledge of the pertinent precursor of desoxyribonucleic acid P. We meet, however, very different conditions when faced with the task to calculate the rate of renewal of a carbon labelled compound from specific activity or similar data.

C^{14} of rapidly metabolized carbon compounds such as acetate, glucose and so on is within a comparatively short time exhaled to a very large extent as carbon dioxide. Correspondingly the rate of loss of C^{14} by fatty acid molecules in a later phase of the experiment in which, for example, labelled acetate was administered to the rat, is no longer a resultant of the disappearance of "old" strongly labelled molecules and the formation of "new" less strongly labelled ones but, at least in the first approximation, the result of the decay of labelled molecules only. By following the rate of decrease of the C^{14} content of fatty acids extracted from the organs of the rat we can thus calculate the renewal rate of fatty acid molecules in an analogous way to that, in which we calculate the period of decay of a radioactive body from its "decay curve." In the present note the calculation of the renewal rate of the fatty acids of the liver from the rate of decrease of the C^{14} content of the fatty acids in the rat injected with acetate labelled in the carboxyl group is described.

EXPERIMENTAL

44 rats weighing 190 to 244 gm were injected intraperitoneally each with 0.2 ml of phys. sodium chloride solution containing acetate labelled in the carboxyl group of 10–26 μ C activity. The animals were killed by decapitation and the total fat,

[1] Recent results obtained by Ottesen (7) and by Abrams (1) indicate that inorganic P is the pertinent precursor of desoxyribonucleic acid P.

fatty acids, neutral and phosphatide fatty acids, and also cholesterol of the liver, secured as described previously (2, 8). The activity of the samples was compared without conversion into barium carbonate.

RESULTS

In an early investigation, using deuterium as an indicator, Stetten and Boxer (12) found the half life-time of fatty acids of the rat liver to amount to 1.9 days. Rittenberg and Bloch (11), feeding C^{13} labelled acetate, observed a more rapid incorporation of C^{13} into saturated than into unsaturated fatty acids of the mouse liver. In a more recent work, Pihl *et al.* (8, 9) compared the C^{14} content of the fatty acid carbon (at the end of the experiment taking many days) with the average value of the C^{14} content of the precursor carbon prevailing during the experiment. The saturated fatty aids of the liver were found to reach half of their maximum isotope concentration in less than one day, the unsaturated acids in about two days.

When investigating the effect of muscular exercise on the incorporation of C^{14} into liver fats, the present writer (5) found after the lapse of 4 ½ hr the C^{14} content to be only one third of that detected 20 min. after injecting labelled acetate into the mouse; however, according to the above mentioned data, in the course of two hr. the loss of C^{14} by liver fatty acids should be less than 10 per cent. Obviously, a fatty fraction present in the liver must have a much shorter half life-time than one day. Ruyssen, Beeckmans and the author (6) investigated the renewal rate of fatty acids in the liver of the mouse shortly after administration of labelled acetate. They obtained the result that the C^{14} content of the liver fatty acids increase rapidly during about the first 30 minutes, this increase being followed by a rapid decrease. Furthermore, Beeckmans and Elliott (2) could demonstrate that in both the saturated and unsaturated fatty acid fractions this early increase is followed by a rapid decline in the C^{14} content. The liver must thus contain one or more rapidly renewed fatty acid fractions. To discard the effect of this rapidly metabolizing fatty acid fraction we studied the decrease in the C^{14} content of liver fatty acids one or 1 ¼ days after administration of labelled acetate only and followed it till the lapse of 4 or 4 ¼ days. As we in our determination of the renewal rate disregard those labelled fatty acid molecules which are formed during the experiment we have obviously quite apart from the above considerations to wait for about 1 day after injecting labelled acetate before securing the samples.

Gould *et al.* (4) found that after the lapse of three hr. only 85 per cent of the acetate C^{14} injected into the rat is already exhaled as $C^{14}O_2$. In the later phase

of the experiment the rapid loss of the exogenous acetate C^{14} may to some extent be compensated by formation of labelled endogenous acetate. Pihl *et al.* (9) kept the activity of body acetate of the rat at a constant level by feeding labelled acetate; in these experiments an increase in the body acetate activity could be observed after the lapse of ten days which was due to the formation of endogenous acetate of appreciable C^{14} content. Under our experimental conditions (injection of the labelled acetate at the start of the experiment) the activity of endogenous acetate formed during the experiment was, however, negligible.

In Fig. 1 the decrease in the specific activity of fatty acid carbon with time $1\frac{1}{4}$ to $4\frac{1}{4}$ days after injection of labelled acetate is plotted; also data obtained by Pihl *et al.* (9) for the increase in the percentage of labelled fatty acids with time are plotted. The figure contains thus both "rise curves"and "decay curves." From the data of Pihl *et al.* follow that the half life-time of saturated fatty acids is less than one day and that of unsaturated fatty acids about two days; our data indicate practically the same result, 0.8 and 2.2 days, respectively. A closer coincidence is hardly to be expected in view of the fact that even when comparing fatty acid turnover in the liver of rats of the same race, age and weight, very appreciable fluctuations appear. Amelioration of purification or measuring methods would hardly lead to more accurate mean renewal times, such could be obtained only by investigating an appreciably larger number of animals.

The significance of the data obtained is restricted, as both the saturated and unsaturated fatty acids represent a mixture of components having different turnover rates, some components of the unsaturated fatty acid mixture as linoleic or linolenic acid are not synthesized in the animal organism, and thus are not labelled. While Pihl and Bloch (8) state that the linolenic acid content of the rat liver is almost negligible, these authors find neutral fatty acids to contain 16 per cent, phosphatide fatty acids 8 per cent of linoleic acid. From the fatty acids extracted from the liver of rabbits Popjak and Beeckmans (10) found that when the acetate was given to the animals for 20 hr. the specific activity of phosphatide fatty acids was larger than that of the glyceride fatty acids, in experiments of longer duration, however, no significant difference in the C^{14} content of the two types of fatty acids could be observed.

In all our experiments 1 to 4 days after injecting the labelled acetate the specific activity of the total fatty acids of neutral fat of the rat liver was found to be a few per cent higher than the specific activity of phosphatide fatty acids, while according to Pihl and Bloch (8) 3 days after feeding labelled

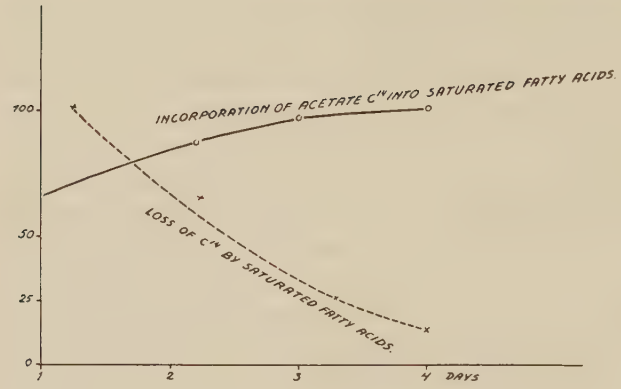


Fig. 1. Rate of incorporation of acetate C^{14} (Pihl *et al.*) and rate of loss of C^{14} by saturated fatty acids extracted from rat liver. ("Rise curve" and "Decay curve.")

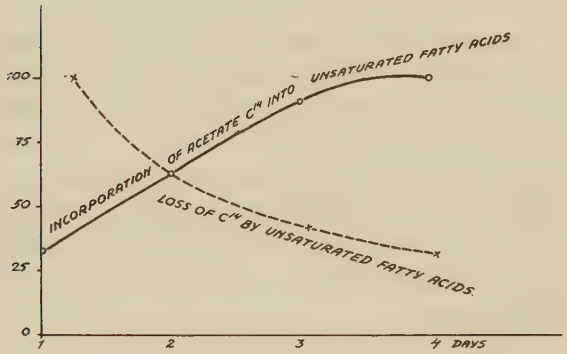


Fig. 2. Rate of incorporation of acetate C^{14} (Pihl *et al.*) and rate of loss of C^{14} by unsaturated fatty acids extracted from rat liver.

acetate to rats kept on lipid free diet, the phosphatide fatty acids are 6 to 13 per cent more active than the total fatty acids of neutral fat.

As mentioned above, investigation of the incorporation of C^{14} into fatty acids of the liver shortly after the administration of labelled acetate to mice revealed the existence of *a rapidly metabolising fatty acid fraction*. A rapid decrease in the C^{14} content of total fat, neutral and phosphatide fatty acids of the liver of the rat is also observed shortly after the administration of labelled acetate but is less pronounced than in the case of the mouse liver. Some results obtained when injecting several rats each of which being killed at a different time after intraperitoneal injection of the labelled acetate are shown in Tables I a) and b).

These and similar results are to be interpreted cautiously, among others because—in contrast to the experiments on mice where pooled livers of several mice were extracted—in the present investigation each point indicates the result obtained when extracting a single liver only. The fatty acid meta-

TABLE I

Percentage injected acetate C^{14} present in 1 mg of neutral total fatty acids of neutral fat extracted from the liver of 182–190 g rats

Time after injection	Percentage injected $\times 10^3$ in 1 mg total fatty acids of neutral fat
a)	
20 min.	37.5
80 »	51.4
110 »	63.0
140 »	21.1
170 »	23.2
240 »	28.3
b)	
20 »	56.2
50 »	66.0
80 »	79.2
110 »	38.0

bolism may strongly vary from animal to animal, even when experimenting with animals of the same race, age and weight. The reason of these variations is inherent partly in the constitution of the livers compared and is presumably partly the result of a difference in the amount and time of food taken in by the rat. A difference of minutes only in the time when the animal last took in food may influence the renewal rate of the fatty acids of the liver.

In spite of the great variations in the rate of C^{14} incorporation into liver fatty acids the results shown in table I and further similar results indicate a more rapid loss of fatty acid C^{14} in the first hours of the experiment than in the later phase, the maximum C^{14} incorporation being observed 1—2 hr. after injecting the acetate. One may be inclined to interpret the rapid decrease in the C^{14} content of the fatty acids during the first hours of the experiment as a consequence of the presence of an impurity in the fatty acid fraction. After purification of the fatty acids or the phosphatides according to Folch and van Slyke's method (3) the decerase was still observable. For the fatty acids of the mouse liver the C^{14} content of the isolated lead salts has furthermore shown a similar time dependency to that of fatty acid mixture (2). Lehninger's copper-calcium method of purification, while increasing the activity figures with 10–20 per cent through removal of less active fractions, did not influence significantly the time dependency of activity figures obtained. The

same applies to the already mentioned purification with colloidal iron hydroxide. The specific activity of phosphatide fatty acids, for example, purified by this method, was increased up to 40 per cent; however, the percentage change in C^{14} content of the fatty acids secured at different times after injecting labelled acetate to the rat was not significantly influenced by the purification process.

SUMMARY

Since most of the C^{14} injected as acetate into the rat is exhaled within a day, incorporation of C^{14} into fatty acids of the liver after that date can be neglected in the first approximation. From the rate of loss of C^{14} by the fatty acids of the liver of rats, to which one or more days previously labelled acetate was administered, the rate of renewal of the fatty acids can thus be calculated. The half life of the saturated fatty acid mixture was found to be 0.8 days, that of the unsaturated fatty acid mixture (1 to 4 days after injection of the labelled acetate) to amount to 2.2 days.

The author's thanks are due Miss Grace de Elliott for her very efficient help, and the Alice and Knut Wallenberg Foundation, and the State Research Council for their kind support of this investigation.

REFERENCES

1. Abrams, R., and Goldinger, J. M., *Arch. Biochem.*, **30**, 261 (1951).
2. Beeckmans, L. M., and Elliot, G., *Nature*, **167**, 200 (1951).
3. Folch, J., and van Slyke, D. D., *Proc. Soc. Exptl. Biol. Med.*, **41**, 514 (1939).
4. Gould, R. G., Sinex, F. M., Rosenberg, I. N., Solomon, A. K., and Hastings, A. B., *J. Biol. Chem.*, **177**, 295 (1949).
5. Hevesy, G., *Nature*, **164**, 1007 (1949).
6. Hevesy, G., Ruyssen, R., and Beeckmans, L. M., *Experientia*, **7**, 144 (1951).
7. Ottesen, J., Personal communication.
8. Pihl, A., and Bloch, K., *J. Biol. Chem.*, **183**, 431 (1950).
9. Pihl, A., Bloch, K., and Anker, H. S., *ibid.*, **183**, 441 (1950).
10. Popjak, G., and Beeckmans, M. L., *Biochem. J.*, **47**, 233 (1950).
11. Rittenberg, D., and Bloch, K., *J. Biol. Chem.*, **160**, 417 (1945).
12. Stetten, Jr., D., and Boxer, G. E., *ibid.*, **155**, 231 (1944).

STUDIES ON DEPOLARIZATION OF LIGHT AT MICROSCOPE LENS SURFACES

I. THE ORIGIN OF STRAY LIGHT BY ROTATION AT THE LENS SURFACES[1]

SHINYA INOUÉ[2]

Department of Biology, Princeton University, Princeton, N. J.

Received June 15, 1951

It is commonly found that only low aperture lenses can be used with the polarization microscope for measuring or detecting small retardations. With low aperture, the field of the microscope can be made very dark with crossed nicols, even with a very intense light source. Under this condition a weakly birefringent object will shine brightly against a dark background.

If, on the other hand, a wide aperture objective is used and the condenser diaphragm is not stopped down, the field no longer remains dark. In this case a birefringent object does not increase its brightness proportionately with the brightness of the field, and so the contrast between the object and its background decreases. Consequently, if the retardation is not large, the birefringent image is obliterated, or nearly so, at higher apertures. For biologists who attempt to study the fine structure of minute cell organelles, this is a very distressing limitation. A high aperture is necessary for good resolution, and yet at the same time the high sensitivity required for detecting weak birefringence can only be attained at low apertures.

Inoué and Dan (2, 3) suggested that the stray light drowning the birefringent image at high apertures arises by depolarization at lens surfaces. This depolarization gives rise to the so called "polarization cross" (fig. 1), observable at the exit pupil of the objective when the ocular is removed, or with a Bertrand lens. The cross can be seen without any object on the stage, and becomes clearer the more intense the light source, and the less defective the optical system.

---

[1] Presented to the faculty of Princeton University in partial fulfilment of the requirements for the degree of Doctor of Philosophy. This research was supported in part by funds of the Eugene Higgins Trust allocated to Princeton University.

[2] Present address: Department of Anatomy, School of Medicine, University of Washington, Seattle 5, Wash., USA.

The existence of the polarization cross means that the extinction is nearly perfect only along the arms of the cross, which are parallel and perpendicular to the axes of the polarizer and the analyzer. Stray light enters into the system from the bright regions between the dark arms.

Now, the polarization cross is similar in appearance to the interference figures of monaxial crystals, but is caused in an entirely different manner. Rinne (5) first explained the cross to be caused by rotation of the plane of polarization at lens surfaces. Each of the rays which hits the lenses obliquely is considered to be separated into two vectors at every glass surface, one parallel and the other perpendicular to the plane of incidence. The two vectors are reflected at different rates, and so the two transmitted vectors no longer have the same ratio as the two entrant vectors. The combined transmitted vector, therefore, has a different direction compared to the original entrant vector (fig. 2). This results in a rotation, whose direction in each quadrant of the cross is determinate, since the vector perpendicular to the plane of incidence always suffers the greater reflection. The amount of rotation is greater the greater the angle of incidence, and the greater the angle between the plane of polarization and the plane of incidence.

Rinne's explanation was first confirmed and amplified by Césaro (1) and later by Wright (6, 7, 8). Using Fresnel's formulae both Césaro and Wright calculated the expected rotation for rays passing through tilted glass plates, as well as the amount of rotation at different points of the exit pupil of an idealized objective lens. Their calculations agree well with direct measurements of rotation, but Wright and others have suggested that rotation may not be the only cause of depolarization at high apertures. Therefore, a formula for the total amount of light introduced by rotation alone within given apertures has been developed by the present author to test this suggestion. The new formula will be shown to agree well with the amount of stray light actually measured at various apertures photo-electrically, and so the other possible factors considered by Wright to cause depolarization must be quite insignificant. The argument is as follows.

Between crossed nicols, the amount of light L introduced by a transparent plate whose area is A, and which has a rotation of Θ is

$$L = AI_0 \sin^2 \Theta \quad \ldots\ldots\ldots\ldots\ldots \quad [1]$$

where I_0 is the intensity (per unit area) of light that passes through the system when the nicols are parallel and when there is no rotation.

Knowing the amount of rotation of rays which pass through the different points of the objective exit pupil, it is possible to calculate from equation [1],

the amount of stray light which would enter into the system by rotation alone within any given aperture.

Since the formulae given by Césaro and Wright are not suited for integration, approximate formulae have been developed as shown below.

CALCULATION OF THE ROTATION OF RAYS PASSING THROUGH DIFFERENT POINTS OF THE EXIT PUPIL

In the following discussions it will be assumed that "critical" (namely, Kohler's) illumination is being used. It is also assumed that the field diaphragm (Df) is made so small that all of the rays emerging from the condenser (Lc) converge into a small point at the center of the object plane (Po). Under this condition, the path of light through the microscope can be simply expressed as in fig. 3.

It is clear from fig. 3 that, if the nicols are not crossed, a solid cone of light would appear as a bright disc at the exit pupil (D_0) of the objective. The radius (r) of this disc has been shown by Mallard (4) and later by Wright (7, p. 148), to be closely proportional to the sine of the half angle Θ of the cone. Since sin Θ is the angular aperture, by studying the optical phenomena at the exit pupil of the objective, it is possible to determine what happens to each of the rays of light passing through the object plane at different angles.

Mallard's relation has been tested in the present optical system by taking two objectives with different apertures and using them alternatively in place of the condenser. The angles of the cones of light which were formed by these lenses were measured carefully and plotted against the diameter of the bright disc at the objective exit pupil. As shown in fig. 4, the agreement with Mallard's relation is very close.

The Mallard relation can be expressed graphically as in fig. 5. Here, all the beams of light pass through the center of the sphere *O*, and change their direction at its surface *O-MPA*, so that the resultant beam (*CG*) is approximately parallel to the axis of the system *OM*.

If we consider a beam *OC* which makes an angle Θ with the axis *OM* and intercepts the sphere at *C*, and draw a vertical line *CI* from *C* to plane *OPA*, calling the intercept *I*, then in triangle *COI*,

$$< CIO = < R = < MOI \quad \text{and so} \quad < OCI = < MOC = \Theta.$$

And so $OI = OC \sin \Theta$.

Therefore, the sphere *O-MPA* can be used to represent the exit pupil of the objective, while its center *O* would be the center of the object plane.

If the sphere were to be observed from an infinite distance in the direction OM, the radius of a point C on the sphere, or at the exit pupil, would be equal to the projection (OI) of MC on to plane OPA, and hence equal to $OC \cdot \sin \Theta$.

In this way, rays passing through the object plane at angles between Θ and $\Theta + d\Theta$ will emerge at the exit pupil as a ring whose radius is r to $r + dr_\theta$. The amount of rotation for rays emerging at each point along this ring shall first be considered.

In fig. 5, let POP' and AOA' include the planes of polarization of the polarizer and the analyzer respectively. The two simultaneously are at right angles to each other and to OM.

All of the optical surfaces present in a microscope are spherical and have their centers along OM, and so for a beam of light passing through O and emerging at C, the plane of incidence, refraction and reflection at every surface is always $MOBC$. The angle Φ between OP and $MOBC$ is the azimuth angle for the entrant plane polarized light. Even though the change of the plane of polarization actually occurs at several different surfaces in a microscope, it can be considered to take place in one step at point C.

In figs. 5 and 6, let CD be the vector of the plane polarized light emerging from the polarizer. This is separated into CE and CF at C; CE being perpendicular to the plane of incidence $MOBC$, and CF being in the plane.

Let the reflection for component CE be $E'E$ and for CF, $F'F$. Since the reflectivity for both components is determined only by the angle of incidence Θ and is not affected by the azimuth angle φ, for a constant angle of incidence,

$$EE' = k_n \cdot CE \quad \& \quad FF' = k_p \cdot CF$$

where $k_n \geq k_p$.

The transmitted vectors are

$$CE - E'E = CE' \quad \& \quad CF - F'F = CF'$$

and so the resulting vector CQ is

$$CQ = CE' + CF'.$$

The azimuth Φ' of this vector is no longer the same as the azimuth Φ of the entrant vector CD, for the ratio CE/CF is usually not equal to CE'/CF'; in fact this describes the rotation.

The angle of rotation $(\Phi - \Phi')$ is

$$\Phi - \Phi' = QS/CS, \quad QS = QT \cdot \cos \Phi$$

and
$$QT = QR - TR = k_n \cdot CD \cdot \sin \Phi - k_p \cdot CD \cdot \cos \Phi \cdot \tan \Phi$$
$$= CD\,(k_n - k_p) \sin \Phi$$
$$\therefore QS = CD\,(k_n - k_p)\, \sin \Phi \cdot \cos \Phi.$$

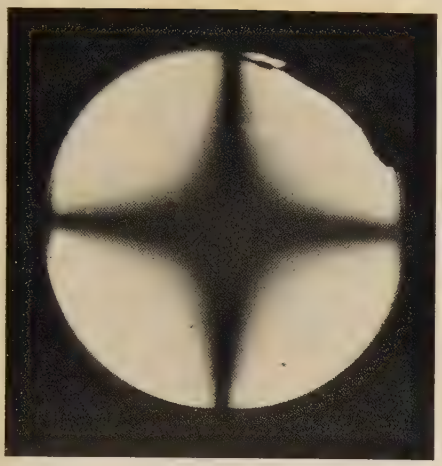


Fig. 1.

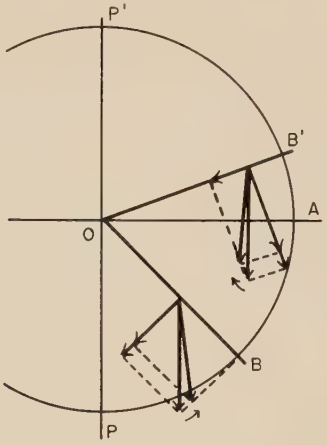


Fig. 2.

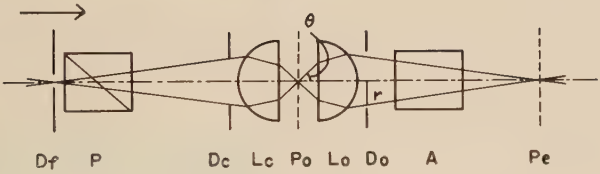


Fig. 3.

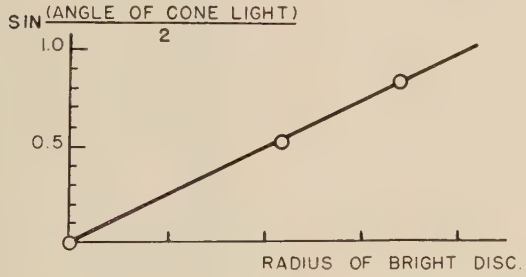


Fig. 4.

Fig. 1. Polarization cross at exit pupil of 4 mm, 0.84 N.A. strain free, coated objective; condenser also strain free lens but not coated. The irregular margin of the upper right quadrant resulted from imperfect blackening of the interior of the lens mount.

Fig. 2. Mode of rotation at lens surfaces in different quadrants. (Compare with figs. 5 and 6.) OP: plane of polarization, OB: plane of incidence.

Fig. 3. Trace of light under critical illumination with the field diaphragm (Df) closed down. The radius (r) of the bright disk at the exit pupil is proportional to sin Θ (Mallard's relation).

Fig. 4. Test for Mallard's relation.

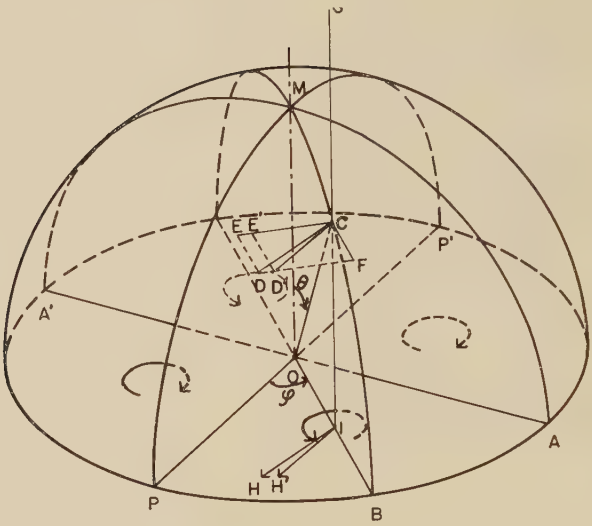


Fig. 5.

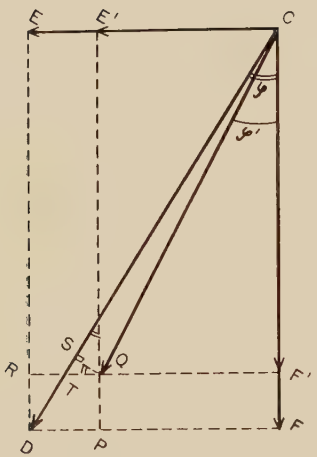


Fig. 6.

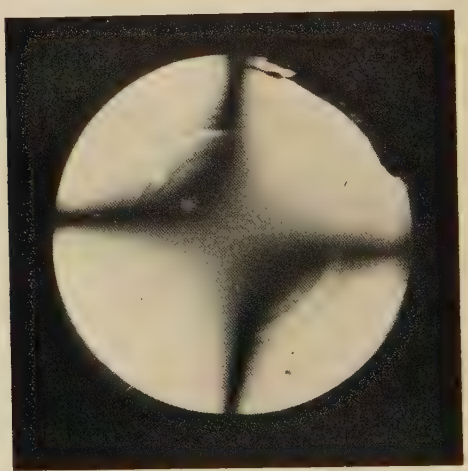


Fig. 7.

Fig. 5. Graphical expression of Mallard's relation.
Fig. 6. Vectorial analysis of rotation. (Compare with Figs. 2 and 5.)
Fig. 7. Appearance of objective exit pupil with the polarizer rotated 0.5° from its crossed position; the condition otherwise the same as in fig. 1.

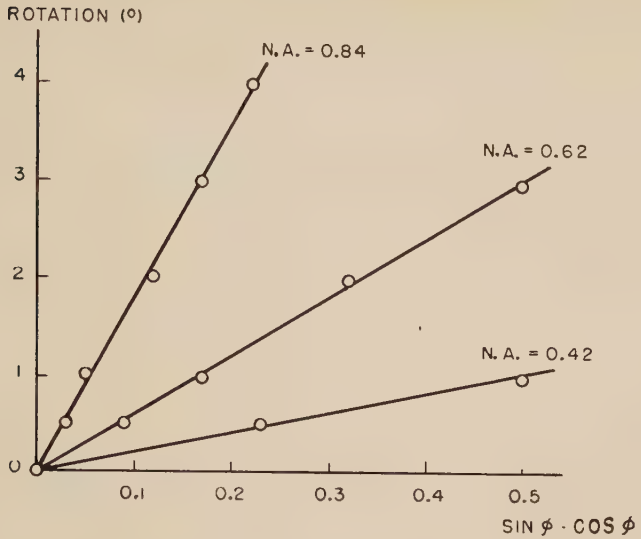


Fig. 8. Rotation measured from photographs (similar to fig. 7) plotted against $\sin \Phi \cos \Phi$. Slope of each curve represents twice the maximum rotation.

Actually $SD << CS$ and so

$$\Phi - \Phi' = \frac{CD\,(k_n - k_p) \cdot \sin \Phi \cdot \cos \Phi}{CD} = f(\Theta) \cdot \sin \Phi \cdot \cos \Phi \ \ldots \quad [2]$$

$(\Phi - \Phi')$ is maximum at $\Phi = \pi/4$ and $\sin \Phi \cos \Phi = 0.5$. Therefore, $f(\Theta)$ is twice the maximum rotation at any given angle of incidence.

The actual amount of rotation can be measured by rotating the polarizer or the analyzer slightly off from the crossed position. Then the polarization cross opens up into two dark hyperbolae (fig. 7) which represent the lines of equal rotation.

In fig. 8, the rotation along certain apertures measured from photographs of the dark hyperbolae are plotted against $\sin \Phi \cdot \cos \Phi$. Clearly within the range of the measurements, the approximate formula very closely describes the relation between rotation and azimuth angles at different angles of incidence.

The maximum rotation at different apertures can be determined from fig.8, for the slope of each line represents $f(\Theta)$ or twice the maximum rotation for each of the corresponding apertures. The log of $f(\Theta)$ determined in this manner has been plotted against Θ, and the relation proves to be perfectly linear (fig. 9). This corresponds to the straight line portion of a sigmoid curve described by Césaro's and Wright's formulae.

The straight line can be expressed as,

$$\log_{10} f(\Theta) - \log f(O) = \frac{\log 10}{0.44} N.A.$$

The value 0.44 was determined graphically from several sets of data and from Césaro's and Wright's formulae. Regardless of the mode of calculation, this and only this universal value was obtained.

Transferring the base of the logarithm to e,

$$0.434 \cdot ln \frac{f(\Theta)}{f(O)} = \frac{N.A.}{0.44}$$

$$f(\Theta) = f(O) e^{N.A./0.19} \quad \ldots\ldots\ldots\ldots \quad [3]$$

Equation [3] is the expression for maximum rotation at different apertures.

THE TOTAL FLUX OF LIGHT INTRODUCED BY ROTATION WITHIN A GIVEN APERTURE

From equations [2] and [3], the rotation $R(r, \Phi)$ for a beam which passes any point (r, Φ) at the exit pupil of the objective is,

$$\begin{aligned} R(r, \Phi) &= f(\Theta) \sin \Phi \cdot \cos \Phi \\ &= f(O) e^{N.A./0.19} \sin \Phi \cdot \cos \Phi. \end{aligned}$$

Using Mallard's relation and by choosing a proper unit for the radius r of the exit pupil of the objective, namely $r = N.A.$,

$$R(r, \Phi) = f(O) e^{r/0.19} \sin \Phi \cdot \cos \Phi.$$

From equation [1], the amount of light introduced by this rotation, is,

$$\begin{aligned} L_{r,\Phi} &= A I_0 \sin^2 \{R(r, \Phi)\} \\ &= A I_0 \sin^2 \{f(O) e^{r/0.19} \sin \Phi \cdot \cos \Phi\} \end{aligned}$$

and since the rotation is smaller than 10 degrees $\sin^2 \Theta = \Theta^2$, and

$$L_{r,\Phi} = A \cdot I_0 \cdot f^2(O) \cdot e^{2r/0.19} \sin^2 \Phi \cdot \cos^2 \Phi.$$

From fig. 10,

$$A = r \, d\Phi \cdot dr$$

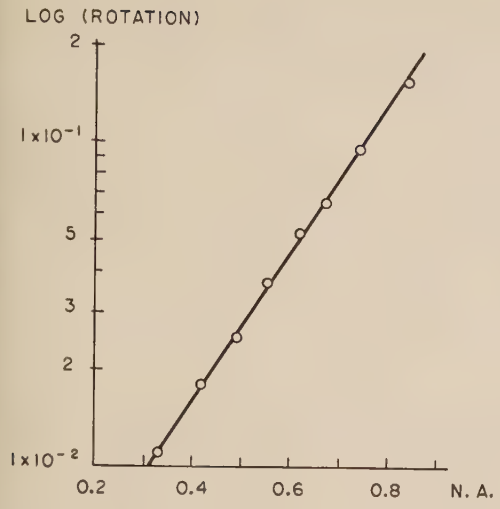


Fig. 9.

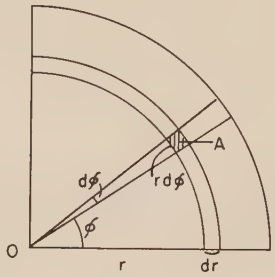


Fig. 10.

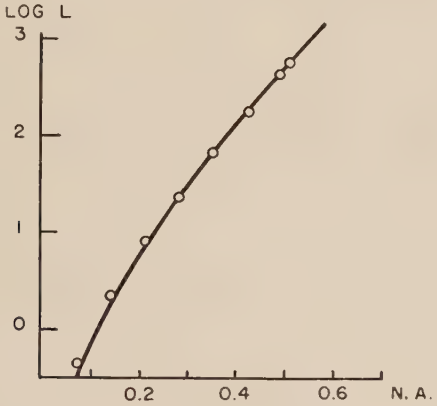


Fig. 11.

Fig. 9. Log (Maximum rotation) plotted against numerical aperture. Maximum rotation was determined graphically from figures similar to fig. 8.
Fig. 10. Construction for integration of amount of stray light introduced at different apertures.
Fig. 11. Curve giving the calculated amount of light (L) entering the system within any given aperture. Circles represent actual photoelectric measurements of stray light. Departure of measurements from calculated values at low aperture can be accounted for; it arises owing to the constant aperture of the objective diaphragms.

and so the total light L is

$$L = I_0 \cdot f^2(O) \int_0^r r \cdot e^{r/.095} \int_0^{2\pi} \sin^2 \Phi \cdot \cos^2 \Phi \cdot d\Phi \cdot dr$$

Since $$\int_0^{2\pi} \sin^2 \Phi \cos^2 \Phi \, d\Phi = \frac{1}{8} \int_0^{4\pi} \sin^2 \Phi \, d\Phi = \frac{\pi}{4}$$

$$L = \frac{I_0 f^2(O)\pi}{4} \int_0^r r \cdot e^{r/.095} \, dr$$

$$= \frac{I_0 f^2(O)\pi}{4} 0.095^2 \left[e^{r/.095} (r/.095 - 1) + 1 \right]_{r=0}^{r}$$

$$L = \frac{9 \cdot 10^{-3} I_0 \cdot f^2(O)\pi}{4} \left\{ e^{r/.095} (r/.095 - 1) + 1 \right\} \quad \ldots\ldots \quad [4]$$

Equation [4] gives the total flux of light introduced by rotation within a given aperture r. Taking the log, we obtain

$$\log L = \log \left\{ \frac{9}{4} I_0 \cdot f^2(O)\pi 10^{-3} \right\} + \log \{ e^{r/.095} (r/.095 - 1) + 1 \} \quad \ldots\ldots \quad [5]$$

The first term on the right is a specific constant for the system, and the second term expresses the change of log L with r, the aperture.

In fig. 11, the line log L (calculated from the equation above) is plotted against r, together with the individual results of direct photo-electric measurements. The very good agreement shows quite definitely that stray light at wider apertures is caused by the rotation of the plane of polarization. The figure also shows very clearly that a small increase in the aperture causes a very great increase in the amount of stray light. Where the aperture is not very small, *a tenfold increase of stray light is brought about by an additional 0.2 numerical aperture.*

This relation then adequately explains how a great amount of stray light is introduced when the numerical aperture of the system is increased in polarization microscopy where the elements are of the usual construction and finish.

REFERENCES

1. Césaro, G., *Bull. Acad. Roy. Méd. Belg.*, 459 (1906).
2. Inoué, S., and Dan, K., *Kagaku*, **19**, 111 (1949) (in Japanese).
3. —— *J. Morphol.*, **89**, (1951).
4. Mallard, E., *Bull. Sci. Min. Fr.*, **5**, 77 (1882).
5. Rinne, F., *Zentr. Mineral. Geol.*, 88 (1900).
6. Wright, F. E., *Am. J. Sci.*, **31**, 157 (1911).
7. ——, The Methods of Petrographic Research. Carnegie Inst. of Washington, Washington, D.C., 1911.
8. —— *J. Optical Soc. Am.*, **7**, 779 (1923).

ON THE MECHANISM OF PHOSPHORYLATIVE ENERGY TRANSFER IN MITOCHONDRIA

O. LINDBERG and L. ERNSTER

Wenner-Grens Institute, University of Stockholm, Sweden

Received August 15, 1951

In recent years, it has been recognized that reactions concerned with aerobic electron transport are localized to certain structural units within the living cell, the mitochondria. Experiments with isolated mitochondria successively led to the development of the cyclophorase concept (9). This concept accounts for the fundamentally important fact that an integrated group of enzymes may possess certain properties and functions which the individual enzymes are unable to exhibit. Thus, the "classical" dehydrogenases and the "organized complex" of these enzymes, differ from each other in that the latter display a coördinated relation with phosphorylation processes (7).

These results have opened the way for a more profound study of the energy generating processes. Whereas the individual enzymatic reactions can be reversed under suitable dismutative conditions, the coördinated mechanism implies a fixation of substrate energy in phosphate bonds. This prevents accumulation of intermediates and renders the overall process irreversible.

When the energy rich phosphate bonds have once been formed, they are transferred by a carrier system through a variety of reactions, until fixed on a final acceptor. The energy generated is thus utilized.

Three phases may be distinguished in the mechanism involved: 1) the formation of the energy phosphate bond, 2) the transphosphorylation reactions, and 3) the utilization ("trapping") of phosphate energy.

The first phase is concerned with the generation of the phosphate bond and is thus coupled directly to electron transport. It includes reactions mediated by carriers, which are presumably common for hydrogen and phosphate and firmly bound to the mitochondria gel. These reactions result in extremely labile primary compounds ("gel phosphate") of an as yet undefined

nature (10). This coupling mechanism is irreversible since hydrogen is fixed by oxygen. Accordingly, the bound carriers become "saturated" and esterification of phosphate ceases (24) when no externally added phosphate carrier is present.

Added adenine nucleotides act as acceptors for the primary phosphate bonds and yield adenosine triphosphate (APPP), thus rendering the energy available for different kinase mechanisms. Adenosine diphosphate (APP) was found to be the kinetically most suitable form of added phosphate transmitter. It has therefore been designated as the "natural" transmitter (3, 14, 23). The question then arises, whether the formation of adenosine triphosphate (APPP) from the primary compound might altogether be ascribed to a simple transphosphorylation mechanism, by analogy with the case of e.g. 1,3-diphosphoglyceric acid. Several facts are, however, opposed to such an assumption. Adenylic acid (AP) can, for example, replace adenosine diphosphate (APP). When, in this case, hexokinase-glucose is added in order to convert the adenosine triphosphate (APPP) formed into adenosine diphosphate (APP), no accumulation of the latter occurs (14). This indicates that an enzymatic interaction takes place in the system between adenylic acid (AP), adenosine di-(APP) and triphosphate (APPP). An explanation of this interaction based on a myokinase reaction

$$2\ \mathrm{APP} \rightleftharpoons \mathrm{APPP} + \mathrm{AP}$$

(3, 14), is not entirely sufficient *This does not explain how adenylic acid (AP) is brought into reaction, when adenosine triphosphate (APPP) is steadily trapped by hexokinase-glucose in excess.*

In a system where no final trapping system is present — as for example in isolated mitochondria, without added hexokinase, — inorganic pyrophosphate (PP) accumulates, often in considerable quantities (7, 11, 16). Little is known about the mode of formation of the inorganic pyrophosphate, but the presence in mitochondria of enzymes catalyzing cleavage of dinucleotides (APPR) into mononucleotides (PR) suggests a metabolic connection between these compounds and pyrophosphate (PP) (9). This connection may be expressed by the following reaction:

$$\mathrm{APPR} + \mathrm{PP} \rightleftharpoons \mathrm{APPP} + \mathrm{PR}.$$

Since this reaction is reversible (15), pyrophosphate cannot be considered as a biologically inert end product. The suggestion, on the other hand, that

pyrophosphate (PP) might be derived from the primary phosphate compound (7), leaves the question open as to whether the pyrophosphate (PP) thus formed reacts according to the above formula. *If this is the case, what mechanism is responsible for the reconstitution of the cleaved dinucleotides in a system supplemented with hexokinase-glucose in excess?*

The aspects considered above suggest that the formation of adenosine triphosphate (APPP) from primary ester and added phosphate transmitter, distinguished above as phase 2), includes a complex pattern of a variety of reactions. The contribution of each of those to the overall mechanism is highly dependent on whether an energy-trapping system (phase 3) is present. Obviously, in the absence of a final acceptor, the individual reactions involved in the formation of adenosine triphosphate (APPP)—such as myokinase or dinucleotidase reaction—run reversibly and accumulations occur. The depletion of the adenosine triphosphate (APPP) formed, on the other hand, determines the direction of these reactions, rendering them irreversible and thus implying a steady break-down of the carrier system involved. A continuity in such a system is hardly conceivable without some kind of internal organization connecting the single steps of generation, transfer and utilization of phosphate energy into a harmonious pattern.

Thus, there seems to be a striking analogy between the principle by which energy fixation in phosphate bonds organizes oxidative processes within mitochondria and the principle by which this mitochondrial function, in its turn, is organized by energy utilization in the cell. This analogy may, from a physiological point of view, seem reasonable. The well known phenomenon that cells, in an active state, often require increased oxygen supply (e.g. working muscle) while, at the same time, adenosine triphosphate (APPP) serves as a source of energy for many of the processes connected with such an active state, indicates a regulation, in some manner, of mitochondrial activity by external stimuli. Moreover, the fact that isolated mitochondria, when supplemented with hexokinase-glucose, exhibit a rate of respiration and phosphorylation superior to that occurring without this supplementation (14, 21), justifies the view that the effect induced by a steady utilization of energy is an essential point when attempting to elucidate the physiological pattern of mitochondrial phosphorylation.

In the present work experimental data were collected in order to gain a better understanding of the mechanism by which kinase processes may regulate oxidation and phosphorylation in the cyclophorase system. The findings obtained led to the advancing of a tentative scheme of this organized pattern.

NOTATIONS

In order to facilitate reference, when writing formulae, to the single phosphate groups within the different compounds concerned, abbreviations differing from the usual ones were employed throughout this report, as tabulated below:

AP = adenylic acid (adenosine-5-phosphate)
APP = adenosine diphosphate
APPP = adenosine triphosphate
APPR = dinucleotide (di- or triphosphopyridine-nucleotide, or flavin-adenine-dinucleotide), PR denoting the respective mononucleotides.
PP = inorganic pyrophosphate
HP = hexose-6-phosphate (equilibrium mixture of glucose- and fructose-6-phosphate).

The site of radioactive labeling is indicated by asterisk, e.g. APP* = adenosine diphosphate labeled in the acid labile phosphate; AP*P = the same compound labeled in the acid stable (ribosidic) phosphate.

MATERIAL AND METHODS

The experiments were performed on rabbit heart mitochondria. The isolated mitochondria were incubated aerobically in the presence of hexokinase and glucose in excess, under conditions varying with respect to added phosphate metabolites. The phosphate turnover of these metabolites was followed, as a function of time, by means of combined isotopic and chromatographic methods.

Preparation of Mitochondria. The procedure by Slater (23) was followed with minor modifications.

Solutions: A) 1.15 per cent KCl (3 vol.), 0.2 M NaF (1 vol.), B) 1.15 per cent KCl (1 vol.), 0.2 M NaF (1 vol.), 0.065 M potassium phosphate, pH 7.5 (2 vol.).

The heart was removed from a decapitated rabbit, immersed in 100 ml of sol. A at 0° C, cut into small pieces with a pair of scissors, and washed by stirring for two minutes. The solution was decanted and the washing repeated four times with new 100 ml portions of sol. A. To the washed heart pieces, 10 grams of quartz sand (Merck analytical quality) and 5 ml of sol. B were added and the mixture was ground in an ice-chilled mortar. The mash was centrifuged in an International Refrigerated Centrifuge at 800 r.p.m. for 2 min. The cloudy supernatant was transferred into a chilled test tube, and the residue was ground again in a mortar with 15 ml of sol. B. This procedure was repeated once. The pooled extracts were centrifuged at 1 000 r.p.m. for a few minutes, in order to remove the last traces of sand and cell fragments. The supernatant was then centrifuged at 18 000

r.p.m. for 15 min. The mitochondria thus obtained were washed twice with small portions of sol. A.

Materials. AP, APP and APPP were commercial products of Sigma Chemical Comp., St. Louis, Mo.

Hexokinase was prepared according to Berger *et al.* (4). The precipitate obtained at step 3a was dissolved in 1 per cent glucose and the solution dialyzed against 1 per cent glucose for about 10 hours before use. The preparation contained hexose phosphate isomerase, but it was completely free from myokinase. The enzyme was freshly prepared for each experiment.

Myokinase was prepared from rat skeletal muscle, according to Colowick and Kalckar (6).

Preparation of Labeled Compounds. Labeled phosphorus in the form of carrier-free $H_3P^{32}O_4$ was obtained from the U.S. Atomic Energy Commission. The samples were hydrolyzed in normal HCl at 100° C for 10 min. before use.

*P*P** was prepared by heating $Na_2HP^*O_4$ of suitable spec. activity at 240° C for 24 hours, according to the classical method recently adopted by Kornberg and Pricer (17).

*APP**. The liver from a starved rabbit was homogenized in a Turmix blender, in 160 ml of a solution containing KCl (0.12 M), glycylglycine buffer pH 7.7 (0.006 M), orthophosphate (0.04 M) and glucose (0.04 M). 50 ml of this homogenate, supplemented with Mg^{++} (0.004 M) and fluoride (0.04 M), was incubated under aeration at 30° C for 2½ min., after which 5 micromoles of orthophosphate containing 5 mc P^{32} were added and the incubation continued for a further period of 4 minutes. The sample was rapidly cooled, fixed by the addition of 15 grams of solid TCA, and filtered. The clear extract was shaken 3 times with ether, in order to remove the bulk of TCA, and the ether expelled by aeration.

The following procedure is based upon a precipitation of the APP* as the cobaltous salt and the removal of the precipitating Co^{++}, as a rhodanide complex, by shaking the sample with organic solvents. The method is advantageous, when small amounts of highly labeled substances are to be separated, in which case the removal of the precipitating metal ions by precipitation—as e.g. the sulfide—may cause serious losses by adsorption; furthermore, the substances to be separated are here obtained as a mixture of the NH_4-salts, free from inorganic salts, and suitable for continued purification by paper chromatography.

To each 10 ml of the clear extract 0.1 ml 1 M ammonium acetate and 0.1 ml 1 M cobaltous nitrate were added and the pH of the solution was adjusted to 6.8–7.0 by careful addition of 1 M NaOH. A pink precipitate was obtained (a blue one indicates the precipitation of cobaltous hydroxide and is to be avoided). After centrifugation, the precipitate was washed with a few ml of water and dissolved in 5–10 ml 20 per cent TCA. The solution was saturated with solid ammonium rhodanide and shaken 4 times with 25 ml of a mixture of equal volumes of amyl alcohol and ether. Hereby, the cobalt migrates into the organic phase as a deep-blue rhodanide complex. The colourless water solution was shaken several times with ether in order to remove dissolved amyl alcohol, and, after having adjusted

the pH to 7 with diluted ammonia, evaporated to dryness over sulfuric acid. The dry cake, consisting mainly of ammonium rhodanide, was suspended in water-free acetone, whereby the rhodanide goes into solution, while the minute residue contains the APP* to be isolated.

The residue was dried, dissolved in a minimum volume of water and placed, in the form of a row of spots, on a sheet of filter paper. On each spot, in addition, 5 microliters of a 0.01 M APP solution were placed. Chromatography was performed according to the method cited below, the sheet being rinsed in abs. ethanol before drying. A strip including the APP-spots as recognized in the light of a metallic lamp, was cut out and extracted with a few ml of water. The purity of the substance was tested by repeated chromatography on a minute aliquot of the sample.

*AP*P.* The synthesis included the following steps: A) incubation of baker's yeast, under aerobic conditions, in the presence of labeled orthophosphate; isolation from the yeast of diphosphopyridine nucleotide, which in this way becomes labeled (25).

B) incubation of the isolated dinucleotide with respiring kidney mitochondria in the presence of unlabeled orthophosphate. The dinucleotide undergoes cleavage and the resulting AP* becomes phosphorylated to AP*PP (16).

C) the AP*PP, isolated, is converted into AP*P by incubation with hexokinase and glucose.

A) 20 grams of baker's yeast were cultivated under aerobic conditions, as described elsewhere (25), in the presence of 8 mc P^{32} as Na_2HPO_4, for two hours. The suspension was filtered on high-flow by suction. After washing with water, the yeast-high-flow-cake was transferred into 50 ml of boiling water and extracted at 92° C for two min. To the extract, 40 grams/100 ml of solid ammonium sulfate and a few drops of 10-n H_2SO_4 were added and the solution was shaken with 30 g phenol. After centrifugation, the phenol phase was sucked off and the treatment repeated with 10 g phenol. The combined phenol extracts were washed twice with small amounts of water, after which 5 volumes of ether were added and the phenol-ether mixture was shaken with a few ml of water. The water solution thus obtained contains a relatively pure mixture of dinucleotides, the bulk of which consists of labeled diphosphopyridine nucleotide.

B) Kidney cortex mitochondria from a rabbit were prepared in the same way as described previously for heart preparation, and suspended in a solution containing the usual set-up of additions for aerobic incubation (cf. "Incubation procedure"). The dinucleotide preparation obtained above was employed as a phosphate transmitter. The suspension, amounting to a volume of about 10 ml, was incubated in O_2-atmosphere at 30° C for 15 min.

C) After fixation with TCA, the extract was treated with Co^{++} as described above, and AP*PP purified chromatographically. The solution of AP*PP obtained was incubated with hexokinase in the presence of glucose, in order to form AP*P.

The localization of labeling in the substance obtained was tested chromatographically.

Incubation Procedure. The mitochondria were suspended in a mixture of 5 ml of 1.15 per cent KCl and 0.5 ml 0.2 M glycylglycine buffer (pH = 7.5). As standard

additions, glutamate, Mg^{++} and NaF were added, in final concentrations of 40, 4 and 30 micromoles/ml, respectively. Orthophosphate, AP, APP, PP, glucose and hexokinase were added according to the different experimental conditions, as indicated in each case. A final volume of about 10 ml was obtained.

Incubations were carried out in wide test tubes (36 × 115 mm) placed in a water bath of 30° C. A stream of oxygen was blown through the suspension.

Treatment of Samples. Samples of 0.2 ml were removed, at suitable intervals, by means of a pipette, graduated at 0.1 and 0.2 ml.

0.1 ml of the removed sample was fixed in 0.1 ml isobutyric acid containing 1.5 ml conc. aqueous ammonia/100 ml, and kept in the frozen state until subjected to chromatography.

The remaining 0.1 ml was employed for determination of orthophosphate according to Martin and Doty (20). The sample was pipetted into a test-tube containing 3.4 ml 0.94-n H_2SO_4, 1 ml silicotungstic acid and 5 ml isobutanol-benzene (1:1). After the addition of 0.5 ml 10 per cent ammonium molybdate, the mixture was shaken and phosphate determined photometrically in an aliquot of the isobutanol-benzene-phase. As the whole procedure can be performed within a few minutes, it renders possible an estimation, *during* the incubation, of phosphate uptake.

Analytical Methods. The samples fixed in isobutyric acid were analyzed with respect to phosphorous derivatives by paper chromatography according to Zetterström and Ljunggren (26). The radioactivity of the different compounds on the chromatograms was thereafter measured *in situ*, by the method of Lindberg and Hummel (19).

The frozen samples were allowed to thaw and were mixed, by a stream of air from a capillary pipette, until a homogenous suspension was obtained. About 5 microliters of each sample were pipetted onto a sheet of filter paper (26 × 49 cm), to form a series of spots at two cm intervals along a line drawn 6 cm from the end of the sheet. In order to obtain points of reference after partition, 5 microliters of a mixture of APP and APPP (equal parts of 0.02 M solutions) had been previously applied at the location of each spot.

After the spots had dried, the sheets were hung in all-glass containers of suitable size and the chromatograms "developed", in a solvent consisting of isobutyric acid, water and ammonia, for 8–12 hours. The wet papers were washed twice by dipping for a few minutes in absolute ethanol and dried in an air stream. Partition was tested by localizing the control spots of APP and APPP in the light from a metallic lamp. After marking out these points with a pencil, the sheets were cut into strips of 2 cm width, each strip including one application point and the corresponding spots of control substances.

Radioactivity was measured in an electronic counter of commercial type, by pulling the strip under the GM tube and registering the number of counts/min. for each half centimeter interval. The sum of the values comprised in each peak was computed in terms of percentage of the total number of counts/min. on the strip. In the subsequent discussion this value will be referred to as P.C.A. (per cent activity).

TABLE I

Compound	$RF_{rel.}$ (mean value for spot, middle)	Test of identity
Inorganic pyrophosphate	38.4	Extraction of the spot in the presence of added carrier and repeated crystallization as the Cd-salt until constant specific activity obtained (5)
Hexose-6-phosphate		
Glucose-6-phosphate	56.7	Determination of hydrolysis rate (2)
Fructose-6-phosphate	66.1	
Orthophosphate	78.1	
Adenosine triphosphate	81.7	Parallel chromatogram after incubation of the sample with hexokinase-glucose. The original APPP-peak hereby recovered as an increase in APP- and HP-peaks.
Adenosine diphosphate	100.0[1]	Parallel chromatogram after previous incubation of the sample with myokinase and hexokinase-glucose, APP-peak hereby becoming extinct.
Adenylic acid	129.0	

Chromatographic separation and identification of phosphorus derivatives occurring in mitochondria suspensions after aerobic incubation in the presence of hexokinase-glucose and fixation in isobutyric acid.

The identification of the different peaks was performed, both by comparing the obtained RF-values with those of respective substances in pure form, and by specific chemical and/or enzymatic assays, as summarized in Table I. These tests were likewise applied in cases where an overlapping of two peaks occurred. This was of special importance in discerning APPP from orthophosphate, the two compounds having very close RF-values in the system employed. This was achieved by running two parallel chromatograms with the same sample, one of them having previously been incubated with hexokinase-glucose.

In such cases, furthermore, and especially in the experiments with labeled intermediates (cf. Exps. III and IV), information in addition to the chromatographic values could be obtained by determination of the radioactivity of orthophosphate, acid labile phosphate and phosphate stabile to acid hydrolysis. The method of Martin and Doty, as adopted for isotopic use by Ernster *et al.* (8), was used. For this purpose, the remainder of the sample fixed in isobutyric acid was diluted with

[1] $RF_{rel.}=100$ corresponds to an absolute RF-value of about 0.4–0.5.

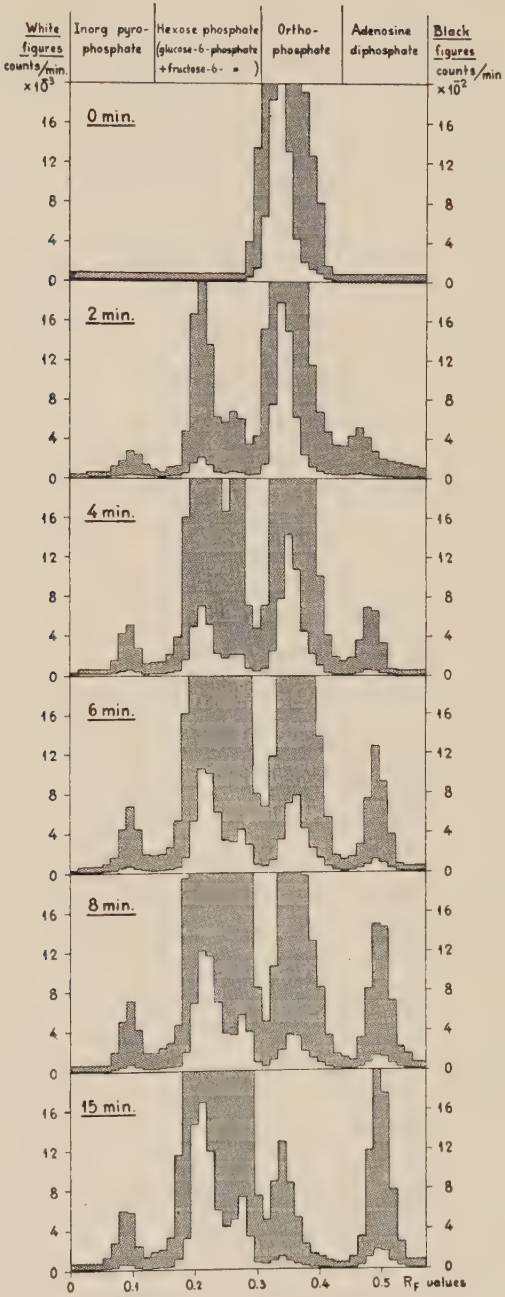


Fig. 1. Mitochondria from one rabbit heart were incubated in 10 ml of a mixture containing: KCl 0.077 M, glycylglycine buff. pH 7.5 0.01 M, glutamate 0.04 M, Mg^{++} 0.004 M, fluoride 0.03 M, $P^{32}O_4^{---}$ 0.25 mc 0.011 M, AP 0.0013 M, hexokinase in excess, at 30° C in O_2 atmosphere. The samples removed were fixed in isobutyric acid-ammonia and subjected to chromatographic separation. The radioactivity of the different compounds was then determined. The fig. shows results from the chromatographic analyses of samples of mitochondria incubated for different times.

a mixture of 0.85-n H_2SO_4. To 3.5 ml of this solution 0.5 ml 10 per cent ammonium molybdate and 1.0 ml of silicotungstic acid were added. This mixture was then shaken with 5 ml isobutanol-benzene (1:1), and the radioactivity of orthophosphate determined in an aliquot part of the organic layer. Another sample of 3.5 ml vas hydrolyzed in a boiling water bath for 15 min. and thereafter treated as above. The radioactivity was determined in both layers. Thus, the number of counts/min. in ortho-, acid labile, and acid stable phosphate could be computed as a percentage of the total radioactivity present in the sample. These P.C.A. values were compared with the respective ones deduced from the chromatographic data. In Fig. 1, the diagram of a typical experiment is shown.

RESULTS

General Experimental Conditions. The mitochondria prepared in the way described above are free from substrate and other soluble metabolites, the minute amount of acid soluble phosphate present, the bulk of which reacts as orthophosphate, being presumably identical with that denoted by Green *et al.* (10) as "gel phosphate".

As requirements for oxidative phosphorylation, the system is to be supplied with, besides oxygen, an oxidizable substrate, Mg^{++}, orthophosphate and an adenine nucleotide — AP or APP — as a transmitter of the esterified phosphate to a final acceptor. This latter was, in the following experiments, represented by glucose. The system was supplemented with a large excess of hexokinase, capable of degrading APPP, at the same rate as it is formed, into APP and with the simultaneous formation of hexose-6-phosphate (HP). Thus the hexokinase competes with the APPP-ase present in the system. Since, furthermore, no enzymatic break-down of hexose-6-phosphate (HP) was detectable during the period of investigation and since the weak pyrophosphatase present in mitochondria was completely inhibited by fluoride, the system was, in this way, totally devoid of back-reactions leading to a reappearance of orthophosphate.

Under such conditions, the orthophosphate taken up is rapidly transformed into hexose-6-phosphate (HP) and, consequently, any intermediates appearing can be expected to be of a very low order of magnitude as compared with the two former compounds. The method described in the preceding chapter was worked out in order to render it possible to detect these intermediates, follow their turnover and elucidate their relation to each other and to the overall pattern. This method, however, has its weakness in only giving information about the relative amount of labeled phosphorus (per cent activity = P.C.A.) present in the intermediates in question, but *not* about their molar quantity. On the other hand, both these data are

needed in order to evaluate specific activity or turnover rate. Quantitative chemical determinations were difficult, considering the fact that the amount of the different intermediates separated on a paper strip was of an order of magnitude of 10^{-3} micromoles.

The difficulty was overcome by arranging the experimental conditions in a manner which made it possible to deduce the amount of the different compounds from their labeling.

In some of the experiments, this aim was achieved by only adding *one* source of metabolizing phosphate to the system, viz. orthophosphate. As pointed out above, this is indispensable for oxidative phosphorylation. The phosphate transmitter used was AP, the phosphate group of which undergoes no renewal in experiments of this kind (14). Under the assumption that the amount of orthophosphate added and the rate of phosphate uptake (amount of orthophosphate esterified per minute) are sufficiently high as compared with the level of "gel phosphate" present in the system, the amount of the intermediates formed can be calculated when knowing their radioactivity (P.C.A.) and the specific activity of the added orthophosphate.

In other cases, however, it was of interest to investigate the behavior of the different intermediates when present from the beginning of the experiment. Under these conditions, the system becomes supplied with *two* different sources of metabolizing phosphate, i.e. the intermediate in question and orthophosphate. Therefore, a calculation of the amount of phosphate present in the different compounds is no longer possible in the simple way indicated above.

In these experiments, the incubations were performed in duplicate samples, one of them containing the intermediate in labeled form and unlabeled orthophosphate, while the other sample contained the same additions interchanged with respect to labeling (in the following referred to as "double labeling" technique). Knowing the specific activity of the added labeled substance, the contribution of this to any given compound could be calculated in the respective samples. The *sum* of these contributions, then, yielded the amount of the compound in question.[1] Practical applications of this method of calculation are found in Tables III and IV.

Description of Experiments. In *Exp. I*, mitochondria were incubated in the presence of labeled orthophosphate as the only source of metabolizing

[1] Imparities due to small contaminations of labeled orthophosphate possibly occurring in the labeled metabolites could be eliminated by aid of the parallel experiment with labeled orthophosphate.

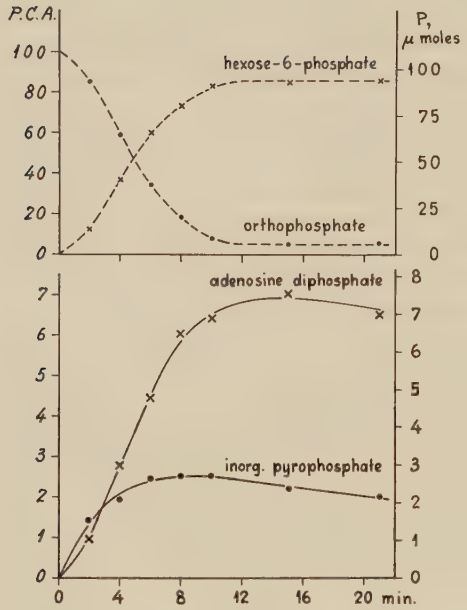


Fig. 2. *Exp. I.* Conditions as in Fig. 1. The different P.C.A. values obtained analytically were converted into corresponding amounts of P by multiplying the respective P.C.A. values by the amount of ortho P present at 0 time/100.

phosphate. AP served as a phosphate transmitter and the APPP formed was steadily "trapped" by the hexokinase added in excess, being thus converted into APP, while, simultaneously, HP was formed. Fig. 2 shows the labeling of the different phosphorus derivatives obtained analytically, as plotted against time. The values can, in accordance with the foregoing reasoning, be converted into micromoles of P, by division by the specific activity of the added orthophosphate.

It is seen from the figure that orthophosphate was taken up at a rather high and uniform rate, except for a short initial period of retardation and a final flagging when orthophosphate was becoming exhausted. The bulk of the esterified phosphate was accumulated, as could be expected, in the form of HP, whereas, at the same time, APP and PP appeared in relatively small amounts, following curves of exponential type, and attaining after a while a constant level. This level tends, however, to decrease when orthophosphate becomes deficient.

Parallel with this experiment, an aliquot part of the mitochondria suspension was incubated without hexokinase. In this sample practically no esterified phosphate of any kind accumulated.

Exp. II was started under the same conditions as *Exp. I.* After APP and

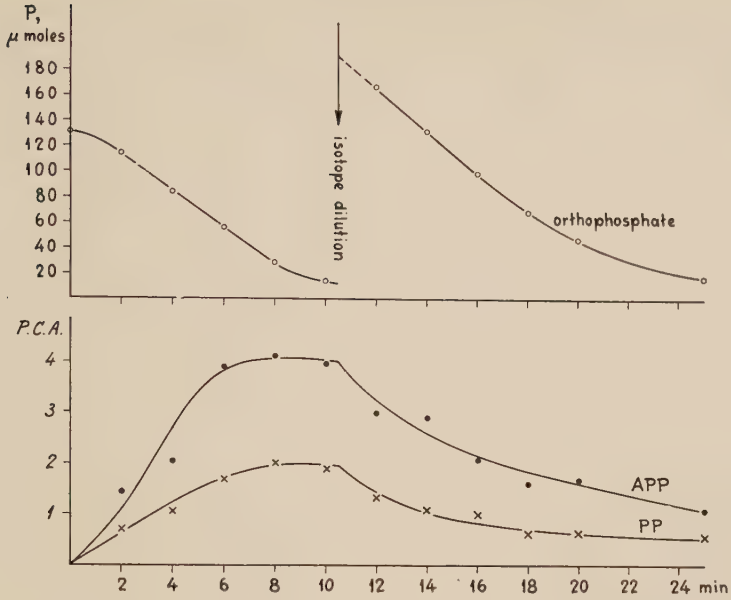


Fig 3. *Exp. II a.* Conditions as in Fig. 1 except the concentration of orthophosphate (0.013 M). After 10 ½ minutes of incubation, unlabeled orthosphate was added in an amount diluting the spec. act. of orthophosphate 15.9 times. The dilution curves of APP and PP serve as a basis for the determination of the respective turnover rates in Table II.

PP had attained constant levels, the incubation mixture was divided into two equal parts, a) and b).

To a) unlabeled orthophosphate was added, in a quantity sufficient to diminish the specific activity of the orthophosphate at least 10 times (a dilution factor of 15.9 was obtained), and the removal of samples continued in ordinary fashion. The aim of this experiment was to determine, by this isotope dilution, the turnover rate of APP and PP and to compare these values with the rate of total phosphate uptake.

In Fig. 3, the upper curves show the course of phosphate uptake as measured by orthophosphate determinations. With regard to the straight-line section of the curve after dilution, a rate of phosphate uptake of

$$\frac{(192\text{–}70)\ \text{micromoles}}{7.5\ \text{min}} = 16.2\ \mu M/min$$

is obtained.

In the lower part of the same figure, the P.C.A.-values for APP and PP are plotted. These values become constant at levels corresponding to 5.24

Table II

t	APP			PP		
	A_t	$S_t = A_t/N$ ($N = 5.24$)	v	A_t	$S_t = A_t/N$ ($N = 2.62$)	v
2	3.05	0.581	0.77	1.32	0.504	0.59
4	2.48	0.473	0.68	0.95	0.363	0.53
6	2.08	0.397	0.63	0.80	0.306	0.45
8	1.79	0.342	0.58	0.70	0.267	0.39
10	1.55	0.296	0.55	0.63	0.241	0.34
12	1.35	0.258	0.54	0.57	0.218	0.32
14	1.15	0.220	0.53	0.52	0.199	0.30
			M = 0.6			M = 0.4

Determination of turnover rate of phosphate in APP and PP by isotope dilution.
Exp. II a (cf. Fig. 3).
Calculation performed according to Eq. 2.
t = time in min. after isotope dilution.
N = amount of metabolite (μM P) calculated from the resp. P.C.A. values before dilution.
A_t = P.C.A. at time t, taken from the dilution curves in Fig. 3.
S_0 and S = spec. act (P.C.A./μM P) of orthophosphate before and after dilution, respectively.
v = turnover rate (μM/min).
The total esterification of P was 16.2 μM/min. c.f. page 221.

and 2.62 micromoles respectively. Assuming that these levels, in accordance with the preceding experiment, were maintained constant during the continued incubation, the turnover rate of the two substances could be calculated, on the basis of the decay of the respective P.C.A.- curves following the isotope dilution, by applying the following formula:

$$\frac{dA_t}{dt} = v(S - S_t) \quad \ldots\ldots\ldots \quad \text{Eq. 1.}$$

or, in explicit form,

$$v = \frac{N}{t} ln \frac{S_0 - S}{S_t - S} \quad \ldots\ldots\ldots \quad \text{Eq. 2.}$$

where t denotes time in min. after dilution; S_0 and S specific activity of orthophosphate before and after dilution, respectively; N the constant level of APP or PP in terms of micromoles of P; A_t and S_t ($= A_t/N$) the P.C.A. value and specific activity, respectively, at time t, of the substance in question; and v, the turnover rate (micromoles/min). In table II, S_t-values

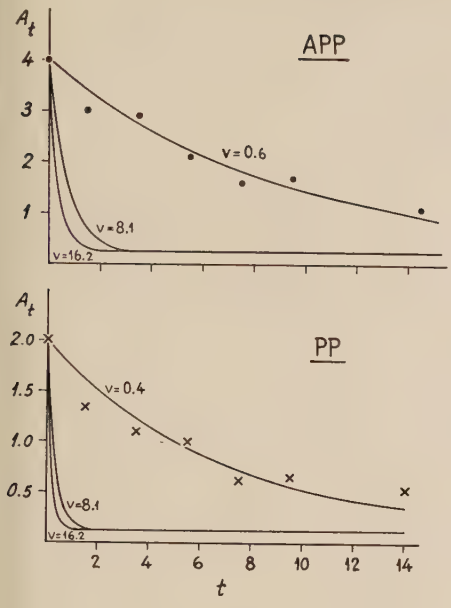


Fig. 4.

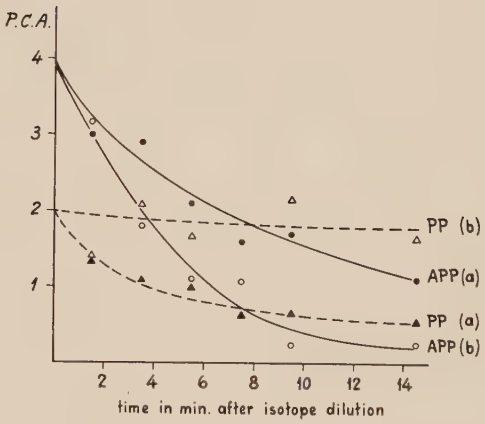


Fig. 5.

Fig. 4. *Exp. II a* (cf. Fig. 3 and Table II). The experimental values from Fig. 3 are compared with the graphs of Eq. 2 when substituting $v = 0.4$ and 0.6 (the mean values obtained in Table II), and also 8.1 and 16.2.

Fig. 5. *Exp. II a, b* (cf. Fig. 3). Effect of added PP on the isotope dilution of PP and APP. (a): dilution curves identical with those in Fig. 3. (b): corresponding dilution curves when isotope dilution was accompanied by the addition of 5 μM PP.

deduced from the experimental curves in Fig. 3 were substituted in Eq. 2, the resulting v-values giving the rough mean-values of

$$v = 0.6 \text{ for APP and}$$
$$v = 0.4 \text{ » PP.}$$

In order to check the significance of these values, the experimental points were compared, in Fig. 4, with the theoretical curves constructed on the basis of Eq. 2, by substituting $v = 0.6$; 16.2; 8.1, in the case of APP, and $v = 0.4$; 16.2; 8.1 in the case of PP. The higher values represent the rate obtained for total phosphate uptake and one-half of this value, respectively.

From this experiment it can thus be concluded that the turnover rates of APP and PP are definitely lower than the rate of total phosphate uptake.

To sample b), the same amount of unlabeled orthophosphate was added as to a), accompanied in b) by a relatively small amount (5 micromoles) of inorganic pyrophosphate. The rate of phosphate uptake was almost

Table III

Compound	Sample	0 min.		½ min.		2 min.		4 min.		6 min.		8 min.		11 min.	
		P.C.A.	μM P	P.C.A.	μM P	P.C.A.	μM P	P.C.A.	μM P	P.C.A.	μM P	P.C.A.	μM P	P.C.A.	μM P
Orthophosphate	(a)	100.0	50.8	98.4	49.9	90.6	45.9	77.1	39.1	65.0	32.9	55.6	28.1	52.3	26.4
	(b)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			50.8		**49.9**		**45.9**		**39.1**		**32.9**		**28.1**		**26.4**
Hexose-6-phosphate	(a)	0	0	0.5	0.3	6.8	3.5	19.1	9.7	30.4	15.4	39.2	19.9	42.9	21.8
	(b)	0	0	21.5	0.9	60.1	2.4	66.7	2.7	68.8	2.8	82.1	3.3	86.3	3.5
			0		**1.2**		**5.9**		**12.4**		**18.2**		**23.2**		**25.3**
Inorganic pyrophosphate	(a)	0	0	0.20	0.10	0.86	0.44	1.51	0.77	1.82	0.93	2.16	1.10	1.66	0.85
	(b)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			0		**0.10**		**0.44**		**0.77**		**0.93**		**1.10**		**0.85**
Adenosine diphosphate	(a)	0	0	0.90	0.46	1.72	0.87	2.32	1.17	2.82	1.43	3.08	1.56	3.18	1.62
	(b)	100	4.00	78.5	3.14	39.9	1.59	32.3	1.33	31.2	1.24	17.9	0.72	13.7	0.55
			4.00		**3.60**		**2.46**		**2.50**		**2.67**		**2.28**		**2.17**

Double labeling experiment with APP.

Exp. III a, b. Conditions as in Fig. 1, except AP was replaced by APP (0.0008 M). Aliquots corresponding to ½ heart contained: (a) ortho-P* and APP; (b) ortho-P and APP*. — P.C.A. values and corresponding amounts of P (μM). The figures set in thick type give the total amount of resp. compounds present.

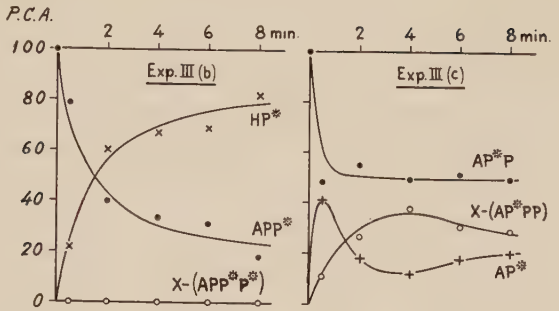


Fig. 6. *Exp. III b, c.* Conditions as in Table III. (b) and (c): APP added as APP* and AP*P respectively. Concerning the symbol X-(APPP) see text.

the same as in a). The curves in Fig. 5 show that the addition of unlabeled pyrophosphate resulted in a decrease in the rate of isotope dilution in PP, demonstrating that "true" PP actually occurs in the system. At the same time, the rate of isotope dilution in APP increased significantly. No quantitative aspects of this effect, however, could be obtained in this experiment, owing to the changes arising in the level of PP and probably likewise in that of APP. For a more systematic elucidation of the effect of added intermediates—both APP and PP—, experiments based upon the double-labeling technique described in the general part of this chapter were performed.

In Exp. III, APP served as a phosphate transmitter in a suspension of mitochondria of otherwise identical composition with that in the preceding experiments. Before the addition of orthophosphate and APP, the suspension was divided into three equal parts, a), b) and c); the above additions were then varied with respect to the site of labeling according to the following scheme:

Sample	*orthophosphate*	*APP*
a)	P*	APP
b)	P	APP*
c)	P	AP*P

Table III gives the P.C.A. values obtained analytically for the different phosphorus derivatives in samples a) and b), respectively. In this table, furthermore, the amounts of the different compounds, originating from the respective labeled source are computed. The *sum* of these contributions, given here in thick type, yields the total amount of the compound present in the sample.

The P.C.A. values obtained in sample c) are plotted in Fig. 6 and com-

pared with those in b). The notation X-(APPP) occurring here refers to a substance to be discussed later.

It is seen from Table III, that the amount of added APP drops sharply until it reaches an approximately constant level. At this time the rates of formation and break-down of APP have attained a common level of about 0.3 micromoles/min., as calculated according to Eq. 2. The total phosphorylation rate was about 5 micromoles/min. Simultaneously with the disappearance of APP, its labile phosphate group is recovered in HP. The AP produced is incorporated into a substance which behaves in the analyses as APPP. Hexokinase and glucose were present in excess in the system, thus trapping *all* APPP at the same rate as it is formed—which is clearly shown in experiment b) where none of the labeled APP appeared as APPP on its way to HP. This shows that the compound behaving in experiment c) as APPP is an artefact. The fixation procedure may cause its splitting from a higher unit which in the following is designated as X-(APPP).[1] The possible existence of this higher unit will be discussed in the following section.

Exp. IV was started in the presence of PP, and with AP as phosphate transmitter. Before the addition of orthophosphate and PP, the incubation mixture was divided into two equal parts a) and b). To a) was added labeled orthophosphate and unlabeled PP, while to b) the same additions were interchanged with respect to labeling.

The results are presented in Table IV, the calculations being performed in a manner analogous to that used in the preceding experiment. In Fig. 7, in addition, the specific activities of the different compounds have been computed by dividing the different P.C.A.-values by the corresponding total amounts of P.

Table IV shows that the amount of added pyrophosphate decreases during the incubation to a constant level. The pyrophosphate which had disappeared was recovered as HP, and APP. The specific activity of the two compounds attains constant levels before that of orthophosphate and PP is stabilized.

Interpretation of Results. In the following section an attempt is made to bring together the mosaic of results presented above into a consistent picture.

[1] In experiments with labeled orthophosphate, the P.C.A. of this substance is too low as compared with that of orthophosphate, which does not render possible its quantitative estimation. Nevertheless, the value for orthophosphate is not significantly influenced by this minute contamination.

TABLE IV

Compound	Sample	0 min.		2 min.		4 min.		6 min.		8 min.		10 min.		12 min.	
		P.C.A.	μM P	P.C.A.	μM P	P.C.A.	μM P	P.C.A.	μM P	P.C.A.	μM P	P.C.A.	μM P	P.C.A.	μM P
Orthophosphate	(a)	100.0	57.0	94.1	53.8	82.9	47.3	72.2	41.4	64.9	37.0	57.8	32.9	48.6	27.8
	(b)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			57.0		**53.8**		**47.3**		**41.4**		**37.0**		**32.9**		**27.8**
Hexose-6-phosphate	(a)	0	0	3.4	1.9	12.8	7.3	22.6	12.9	28.2	16.1	34.5	19.7	43.4	24.7
	(b)	0	0	5.5	0.6	15.4	1.5	26.6	2.7	38.6	3.9	48.5	4.9	51.8	5.2
			0		**2.5**		**8.8**		**15.6**		**20.0**		**24.6**		**29.9**
Inorganic pyrophosphate	(a)	0	0	0.40	0.23	1.09	0.62	1.49	0.85	2.79	1.59	3.30	1.88	4.40	2.51
	(b)	100.0	10.00	93.5	9.35	79.3	7.93	64.8	6.48	51.4	5.14	40.2	4.02	37.5	3.75
			10.00		**9.58**		**8.55**		**7.33**		**6.73**		**5.90**		**6.26**
Adenosine diphosphate	(a)	0	0	0.66	0.38	1.30	0.74	2.28	1.30	2.80	1.60	3.17	1.81	3.06	1.75
	(b)	0	0	1.06	0.11	5.09	0.51	8.11	0.81	10.35	1.04	11.65	1.17	10.75	1.08
			0		**0.49**		**1.25**		**2.11**		**2.64**		**2.98**		**2.83**

Double labeling experiments with PP.

Exp. IV a, b. Incubation mixture as in Fig. 1 supplemented with 10 μm of PP. Suspension divided into two equal parts: (a): ortho P* and PP. (b): orhto P and P*P*. — P.C.A. values and corresponding amounts of P (μM). The figures set in thick type give the total amount of resp. compounds present.

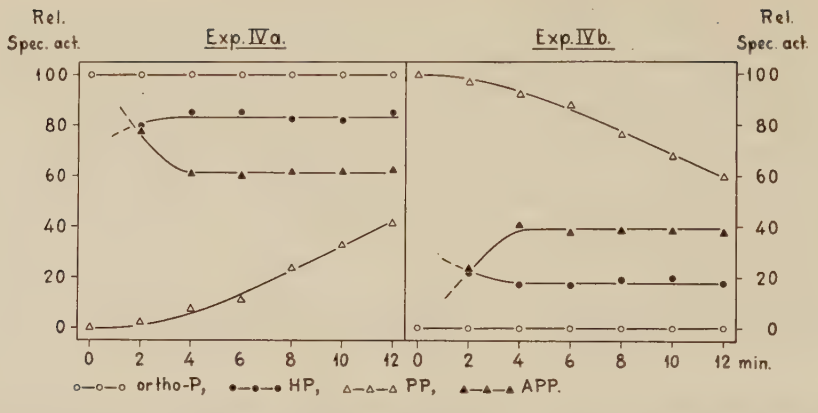


Fig. 7. Rel. spec. act. of different metabolites in Exp. IV a and b. Calculations performed on the basis of Table IV by dividing P.C.A.-values by the resp. total amounts of P (figures set in thick type) and putting the 0-time spec. act. of (a) orthophosphate and of (b) PP, respectively, = 100.

The overall process

$$P \xrightarrow{a} APPP \xrightarrow{b} HP \quad (APP) \qquad (A)$$

will serve as a basis for the following discussion. The symbol *a* designates the entirety of reactions leading from the incorporation of orthophosphate to the formation of APPP, and *b* the hexokinase reaction. As *b* is more rapid in the present experiments, than *a*, an accumulation of APPP will consequently not occur and all reactions involved in *a* go in the direction of the arrow.

Of the resulting products, HP will be piled up while APP is drained off to an extent permitting the maintenance of a constant APP level. Once this level is attained the renewal of APP proceeds at a constant rate. The mechanism for the drainage of APP can be studied in *Exp. III a, b*. By following the destiny of labeled APP, added to the system in excess, it can be inferred that myokinase is responsible for this mechanism:

$$P \xrightarrow{a} APPP \xrightarrow{b} HP \quad AP \leftarrow APP \qquad (B)$$

Obviously, since APPP is steadily trapped by hexokinase, the myokinase cannot explain the formation pattern of APP.

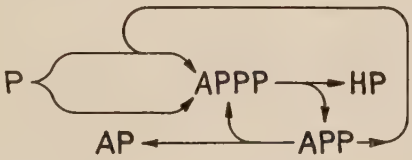
Therefore, the renewal of APP must be involved in process *a*. To what extent this is the case is indicated by experiment II which shows that of every 16.2 micromoles of P passing through *a*, 0.6 micromoles were turned over by APP. The rest must have reached APPP by phosphorylating *pre-existing* APP. Thus, depending on whether or not it involves renewal of APP, the mechanism *a* may be divided into two reaction patterns, one of which is in the following referred to as "the primitive mechanism", giving rise to APPP according to the overall reaction:

(*C*)

and the other "differentiated" one implying the overall reaction:

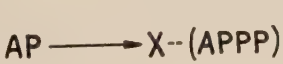
(*D*)

Thus *B* may be written as



(*E*)

In *Exp. III c*, the adenylic acid formed from APP was demonstrated to be incorporated into X-(APPP), which can tentatively be written in the form of the schematic overall reaction

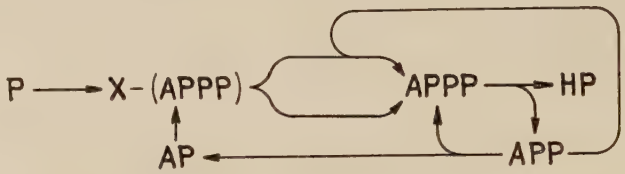


(*F*)

The validity of this reaction formula is very hypothetical, since no further details are known about the compound X-(APPP). It is, at this moment, not possible to decide whether and to what extent it is identical with the

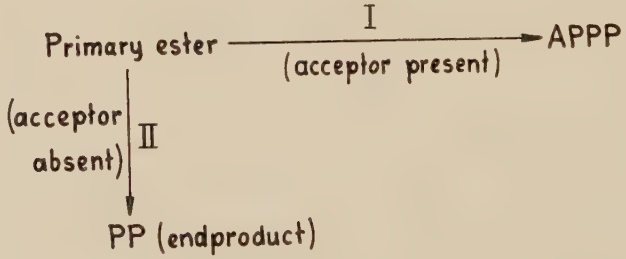
primary ester ("gel-phosphate", Green *et al.* (10)), a part of which, as reported by Albaum (1), actually is recovered after fixation as APPP.

Admittedly, from a functional point of view, it may seem reasonable to draw a parallel between the primary ester and X-(APPP), both constituting an intermediate in the synthesis of APPP.



(*G*)

When characterizing this primary ester, Cross *et al.* (7), advance the hypothesis that this is able to yield PP when the system is devoid of phosphate acceptor, whereas, if acceptor is present, the PP-bond contained in the primary ester is transferred to AP, yielding APPP. This can be summarized as

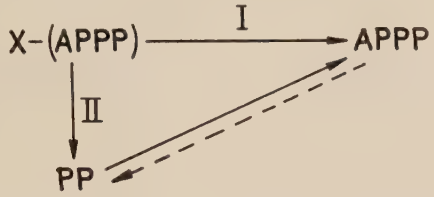


(*H*)

In the present work it could be shown that PP did likewise appear in the presence of a continuous trapping system. Moreover, *Exp II* and *IV* furnish unambiguous evidence for the fact that PP actually is a biologically active intermediate. It is rendered obvious, in this way, that the sharp distinction proposed by Cross *et al.* (7) between "trapped" and "untrapped" systems, with respect to PP-formation, has to be revised. In the absence of hexokinase, PP accumulates in vast amounts; when hexokinase is present, on the other hand, the PP formed is maintained at so low a level that it cannot be identified by the techniques employed by earlier authors (7, 18).

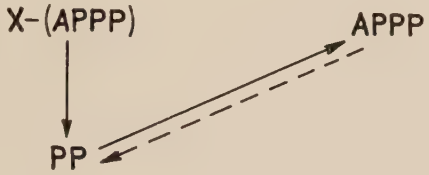
In *Exp. IV*, labeled PP was shown to appear in HP and APP, which indicates that it has passed the labile phosphate groups of APPP. Considering the fact that dinucleotidase is present in mitochondria it would seem

probable that the conversion of PP to APPP is to be ascribed to such a mechanism. We can thus suggest the over all pattern



(*J*)

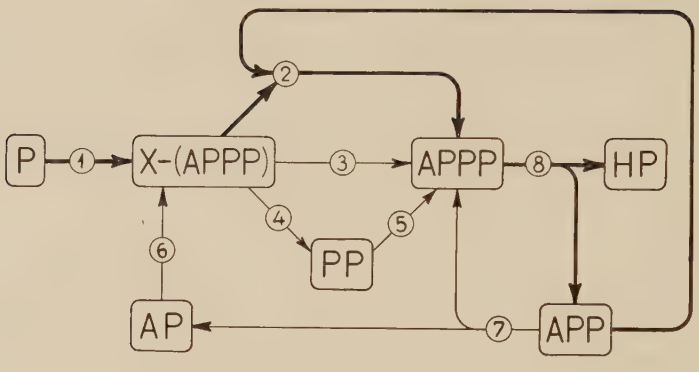
According to such a formulation, it may appear as if X-(APPP) could give rise to APPP in two ways, *viz.* by direct discharging of APPP and by splitting off PP which then is transferred into APPP by a dinucleotidase reaction. The existence of this second pathway, however, renders it questionable whether the presumption of a direct APPP discharge from X-(APPP) is at all justified, or whether this PP-pathway may constitute the only one between X-(APPP) and APPP:



(*K*)

Assuming this latter formulation to be correct, PP would serve as exclusive precursor of the APPP formed by the differentiated mechanism, that is, the only source of APP-renewal. This implies that, in this case, labeling equilibrium should prevail between PP and APP. By introducing labeled PP, in *Exp. IV*, and comparing the courses of specific activity of the added PP and the APP formed (Fig. 7), it became evident that PP cannot be the exclusive precursor of APP. Additional information given by these values is the peculiar constancy of the spec. act. of APP which might indicate that the rate of the two reactions (I and II in formula *J*) are correlated in some manner. Obviously, a deeper knowledge of the chemical nature of X-(APPP) is indispensable for a closer understanding of the mechanism underlying this correlation.

Conclusions. The different steps of the reaction pattern deduced above, can be joined together in the following tentative scheme:



The arrows indicate the direction of the different reactions involved in the combined cyclophorase-hexokinase system. The single reactions are denoted by the numbers 1–8, as referred to in the following description of the general pattern:

Coincidently with the different oxidative steps orthophosphate becomes incorporated into a primary ester (react. 1). This compound was denoted by the symbol X-(APPP), owing to its ability to yield APPP upon acid treatment. From this primary ester, APPP is formed in two ways. One — in the preceding called the primitive pathway — implies a transfer of phosphate from X-(APPP) to APP (react. 2). The second way — tentatively called the differentiated one — involves a splitting of the primary ester.

This occurs partly by direct discharging of APPP (react. 3) and partly by splitting off PP (react. 4) which, in its turn, yields APPP by cleavage of dinucleotides according to the Kornberg reaction (react. 5). In reactions 3 and 4, nothing could be discovered concerning the split products accompanying the formed APPP and PP respectively, nor could the mechanism be determined which is responsible for reconstituting the dinucleotides cleaved in reaction 5. It was emphazised that none of these questions can readily be clarified without further information about the composition of X-(APPP). The only clue as yet obtained experimentally concerning the restoration of X-(APPP) is an incorporation of AP (react. 6).

The APPP formed by either of the above mechanisms is instantly attacked by the hexokinase present and transformed into APP with the simultaneous formation of HP (react. 8). The myokinase present in the cyclophorase system is thus acting in the sense of converting APP furnished by the kinase mechanism into APPP and AP (react. 7).

Table V

Additions			Coupling Capacity a)		Transmitting Capacity b)		Efficiency	
AP	APP	Hexokinase	initial	final	initial	final	initial	final
+	—	—	+ + +	—	—	+ +	+ +	—
+	—	+	+ + +	+ + +	—	+ + +	+	+ + +
—	+	—	+ + +	—	+ + +	+ +	+ + +	—
—	+	+	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +

Influence of different additions on phosphorylating efficiency.
a) Depends upon AP supply.
b) » » APP level.

From a kinetic point of view, the system outlined above is characterized by a correlation of the individual reactions involved, giving rise to a set-up of intermediates — such as PP and APP — which are brought to a level, constant within any given experiment, whether these intermediates were absent at the start or added in excess. Once the levels are established, the rates of formation and breakdown of each intermediate become equal.

In the case of PP, this implies the presence of a mechanism maintaining the rate of reactions 4 and 5 at a common level. In the same way there must be a mechanism correlating the rate of reaction 7 with those of reactions 3 and 4 plus 5 in order to maintain a constant level of APP. In other words the actual rate of the myokinase reaction is determined by the rate of the differentiated mechanism.

The numerical kinetic data calculated in the different experiments afford a possibility to determine the individual contributions of the two main mechanisms to the total APPP production of the system. In *Exp. II*, 0.6 micromoles P passed APP while a total of 16.2 were esterified. In *Exp. III*, the corresponding values were about 0.3 and 5 micromoles per minute respectively. That is, the contribution of the differentiated mechanism amounted in these experiments to about 5 per cent of the total capacity.

This low ratio explains the initial retardation of phosphorylation when starting an experiment with adenylic acid as phosphate transmitter. In other words, in this case the primitive mechanism (requiring pre-existing APP) is limited by the relatively low rate of APP formation through the differentiated mechanism plus hexokinase. When replacing AP by APP phosphorylation proceeds at a uniform rate from the very beginning.

DISCUSSION

In the introduction the hypothesis was formulated that the energy-utilization by kinase processes in the living cell might exert a regulatory effect on the energy-generating function of mitochondria. A comparison of the experimental findings obtained in the present work with earlier data in this field now allows a discussion of these problems from the point of view of the mechanism underlying this physiological correlation.

From a general point of view, the ability of a system to form APPP may be formulated as a resultant of its capacity to generate phosphate bond energy ("*coupling capacity*") and to transmit the energy phosphate generated into the form of APPP ("*transmitting capacity*"). This resultant, denoted as the "*efficiency*" of the system, is then determined by the less efficient of the above two factors. If this *efficiency* is altered in any way, either one or both of these factors must have been affected. Thus, when tracing the way by which the absence of hexokinase affects the rate of oxidation and phosphorylation, one must consider the extent to which the generation of energy phosphate and/or the transmittance of this phosphate into APPP have been altered.

The investigations recently advanced by Kielley and Kielley (14) afford good possibilities to study these problems. These authors have succeeded in obtaining mitochondria preparations devoid of APPP-ase activity. These mitochondria were incubated with and without hexokinase, in both cases with AP as added phosphate transmitter. The sample with hexokinase exhibited a relatively uniform and high rate of phosphorylation, whereas in the system without hexokinase this rate was lower throughout the experiment and declined sharply after a while to reach zero at about 60 minutes. In this sample all the esterified phosphate was accumulated in the form of APPP and APP. The P/O ratio in both cases was unchanged.

In agreement with the view earlier reported by Barkulis and Lehninger (3) and by Slater (23), the authors point out that APP, as the preferred form of phosphate transmitter, is a requirement for optimal phosphorylation. In fact, the data reported in the present work confirm this suggestion by showing that (at least in the case of heart muscle) about 95 per cent of the esterified phosphate has been transferred to glucose by pre-existing APP (*cf. Conclusions*). *The APP level present in a system can thus be regarded as practically determining the actual transmitting capacity.* In experiments without hexokinase, APP is formed by a myokinase reaction. As long as the *transmitting capacity*

is below the *coupling capacity*, this APP formation will consequently be counteracted by the rapid rephosphorylation of APP to APPP. Under such circumstances this lower *transmitting capacity* should be determining the actual *efficiency* of the system. In the experiment cited above, the level of APP — an index of *transmitting capacity* — was only insignificantly changed during the period between 20 and 60 minutes, during which time, however, the rate of phosphorylation — the *efficiency* of the system — diminished rather abruptly from a fairly high level to zero. This striking change in *efficiency* is thus to be attributed, by definition, to a decrease of the *coupling capacity*.

The interpretation given by Kielley and Kielley (14) for explaining the lowered *efficiency* in a system without hexokinase is based upon the statement that an accumulation of APPP may depress further phosphorylation. This explanation fails, however, to elucidate why this depression is already present from the very beginning of the experiment, when APPP had not yet accumulated in appreciable amounts, nor why a relatively small increase in the accumulated amount of APPP in the end period of the experiment is able to induce a total extinction of all further phosphate-uptake.

On the other hand, the fact that this dramatic drop in *efficiency* at the final stage of the experiment coincides, as can be seen from the curves of Kielley and Kielley, with a total depletion of AP from the system, may sooner indicate that these two phenomena are associated with one other. This might indicate that *the supply of AP is an indispensable requirement for the maintenance of the coupling capacity of the system.*

In a system supplemented with hexokinase, an optimal level of APP is reached and maintained (as is shown by the consideration of kinetics given in *Conclusions*) by a correlated contribution of the differentiated mechanism and the myokinase reaction, the latter of which serves at the same time as a continuous producer of AP. It may be concluded, therefore, that the hexokinase increases the *efficiency* of a system by assuring a maximal *transmitting capacity* (an optimal APP level), without simultaneously implying a successive decrease of the *coupling capacity* (AP supply). It is only the *initial transmitting capacity* which is influenced by the circumstance whether the experiment is started with AP or APP. These aspects are diagrammed in Table V.

Thus it would seem that a maximal phosphorylation rate in a system is not only dependent on a level of APP sufficient to transfer, without limitation, the phosphate generated into APPP, but also on a steady supply of AP which apparently is a requirement of the phosphate generating mechanism itself. Actually this suggestion may appear a more concrete formulation

of the view earlier advanced by Cross *et al.* (7) and by Potter (22). According to this, added nucleotides act in the cyclophorase system in a double sense, as transmitters of phosphate and as reconstituents of the coenzyme systems involved in the reactions which couple oxidation and phosphorylation.

We now saw that the former of these functions was mainly carried out by APP, whereas the latter specifically required AP. APP thus seems to play the role of transferring phosphate from the primary ester without having any direct influence on oxidation. The mechanism may be analogous to e.g. the transphosphorylation between 1,3-diphosphoglyceric acid and APP.

AP, on the other hand, may regulate, in some way, the *coupling capacity*, as was postulated above. The mechanism underlying this function of AP is not fully understood. However, it is possible to deduce some information from the experimental data available. In *Conclusions*, it was seen that AP was involved in the reconstitution of the primary ester split by the differentiated mechanism. It might thus seem possible that the core of the problem concerned with the AP effect on the *coupling capacity* is to be found in the differentiated mechanism.

The problem may be approached by posing the question: In which way is AP primarily brought into reaction when added to a system as phosphate transmitter, in the presence or absence of hexokinase? Barkulis and Lehninger (3) and Kielley and Kielley (14) postulate a primary formation of APP by a myokinase reaction, in which connection they recall that small amounts of APPP are present in mitochondria preparations. This interpretation is, however, contradicted by a finding reported by the latter authors. As a consequence of ageing, the mitochondria are deprived of the postulated small supply of APPP. The original initial rate of phosphorylation cannot be restored, however, by addition of the lost amount of APPP.

In our opinion the situation is rather that the initial phosphorylation rate is primarily dependent, as stated in *Conclusions*, on the overall rate of the differentiated mechanism and only secondarily, when some APPP had already been formed through this mechanism, on the myokinase reaction. In the ageing process, it is the primary ester rather than ordinary APPP that becomes degraded (cf. Green *et al.* (10)). For this reason, the differentiated mechanism becomes blocked and cannot readily be restored by the addition of APPP.[1] A similar interpretation is envisaged by Kielley

[1] The fact that Barkulis and Lehninger (3) were able to demonstrate an abolition of phosphorylation when inhibiting myokinase with fluoride does not, according to this reasoning, necessarily indicate that the myokinase reaction is the *primary one* involved.

and Kielley when they point, in this connection, to the active flavinnucleotide found by Hummel and Lindberg (13).[1]

As a matter of fact, there is, at this moment, a body of evidences indicating a connection between dinucleotides and the primary ester (9). The suggestion by Cross *et al.* (7) that the primary ester has the character of a coenzyme pyrophosphate actually gets an experimental support in the present work, though hardly any definite proof is as yet available. It was seen that X-(APPP) is able to split off PP and that this PP, in its turn, reacts with dinucleotides according to the Kornberg reaction. In view of the fact that when associated with the enzyme complex, dinucleotides are not attacked by this cleaving mechanism (12), the possibility might be considered that, *simultaneously* with the formation of PP from the primary ester, dinucleotides become dissociated from their apoenzymes.

By the cleavage of these dissociated dinucleotides an equilibrium tends to prevail, not only between each dinucleotide and its corresponding mononucleotide, but also among the different kinds of di- and mononucleotides, according to the pattern:

$$\begin{aligned} &APPR_I + PP \rightleftharpoons APPP + PR_I \\ &APPP + PR_{II} \rightleftharpoons APPR_{II} + PP \\ \hline &APPR_I + PR_{II} \rightleftharpoons APPR_{II} + PR_I \end{aligned}$$

It is to be noted that, whereas the condition for equilibrium in the two partial reactions involves the presence of both APPP and PP, only one of these substances is needed for the overall equilibrium, and its presence is thus merely of catalytic significance. In this way, if PP is present — as is the case in the system studied in this work —, an equilibrium may be prevailing among dissociated di- and mononucleotides, with the possible consequence that one kind of dinucleotide may determine the degree of dissociation of another.

The significance of this interaction of dinucleotides might be a coördination of the subsequent oxido-phosphorylative steps within oxidase systems with an electron flow mediated by more than one dinucleotide in sequence, e.g. the α-ketoglutarate oxidase including the electron carrier system:

(α-ketoglutarate) → *pyridinedinucleotide* →
flavindinucleotide → cytochrome → (O_2).

[1] The statement made by Green *et al.* (10) that this active flavin compound is too stable to be considered as identical with the primary ester may not wholly exclude the possibility that a flavin containing component of the primary ester might be more stable than those containing other kinds of nucleotides. Its detection may, however, be very difficult when occurring in a mixture along with the more labile components.

According to this reasoning, the mode by which the differentiated mechanism may, as suggested above, regulate the *coupling capacity*, might be explained by such a mechanism.

This explanation, if correct, would imply that, in the case of an oxidase system with only *one* dinucleotide in its chain of electron carriers, oxidation could proceed independently from the differentiated mechanism and would, consequently, require no AP supply. In fact, succinic oxidase, with the electron flow:

$$(\text{succinate}) \rightarrow \textit{flavindinucleotide} \rightarrow \text{cytochrome} \rightarrow (O_2).$$

is independent of AP, also when included in the cyclophorase system (7). Further evidence is, however, necessary to prove the above interpretation.

SUMMARY

The mechanism of phosphorylative energy transfer in mitochondria was studied in relation to the hypothesis that this mechanism is under the physiological control of kinase processes occurring in cytoplasm.

Rabbit heart mitochondria supplemented with hexokinase and glucose were incubated under aerobic conditions with the addition of adenylic acid. The amounts and renewal rates of the phosphate intermediates were studied by means of combined isotopic and chromatographic techniques. Experiments were, furthermore, performed with the addition of these intermediates in labeled form.

The intermediates found were adenosine diphosphate and inorganic pyrophosphate, both these being steadily renewed during adenosine triphosphate formation. Furthermore, evidence for the existence in the working system of a higher nucleotide not identical with any of the known ones was obtained. The compound was characterized by its capacity to yield adenosine triphosphate upon acid treatment. The possibility that this is a primary ester is discussed.

On the basis of kinetic calculations, a tentative scheme has been proposed, including the pattern of reactions leading to the formation of adenosine triphosphate. According to this scheme, adenosine triphosphate is mainly formed through the phosphorylation of adenosine diphosphate by a primary ester. This reaction is referred to as the "primitive mechanism". Another pathway, the "differentiated mechanism" involves the renewal of the primary phosphate carrier, and is connected with dinucleotidase reac-

tions. This mechanism was postulated to be a necessary requirement of coupled phosphorylation.

A mechanism by which kinase processes may influence oxidation and phosphorylation rate in mitochondria is proposed.

This work has been supported by a grant from The Swedish Medical Research Council.

REFERENCES

1. Albaum, H., *Arch. Biochem.*, **24**, 375 (1949).
2. Bamann, E., and Myrbäck, K., Die Methoden d. Fermentforschung. Leipzig, p. 312, 1940.
3. Barkulis, S. S., and Lehninger, A. L., *J. Biol. Chem.*, **190**, 339 (1951).
4. Berger, L., Slein, M. W., Colowick, S. P., and Cori, C. F., *J. Gen. Physiol.*, **29**, 379 (1946).
5. Cohn, G., and Kolthoff, J. M., *Ind. Eng. Chem., Anal. Ed.*, **14**, 886 (1942).
6. Colowick, S. P., and Kalckar, H. M., *J. Biol. Chem.*, **148**, 117 (1943).
7. Cross, R. J., Taggart, J. V., Covo, G. A., and Green, D. E., *ibid.*, **177**, 655 (1949).
8. Ernster, L., Zetterström, R., and Lindberg, O., *Acta Chem. Scand.*, **4**, 942 (1950).
9. Green, D. E., *Biol. Rev.*, **26**, 410 (1951).
10. Green, D. E., Atchley, W. A., Nordmann, J., and Teply, L. J., *Arch. Biochem.*, **24**, 359 (1949).
11. Green, D. E., Loomis, W. F., Dickman, S. R., Auerbach, V. H., and Noyse, B., Abstracts, 111th Meeting Amer. Chem. Soc., Atlantic City, N. J., p. 26 B (1947).
12. Huennekens, F. M., and Green, D. E., *Arch. Biochem.*, **27**, 428 (1950).
13. Hummel, J. P., and Lindberg, O., *J. Biol. Chem.*, **180**, 1 (1949).
14. Kielley, W. W., and Kielley, R. K., *ibid.*, **191**, 485 (1951).
15. Kornberg, A., *ibid.*, **182**, 779 (1950).
16. Kornberg, A., and Lindberg, O., *ibid.*, **176**, 665 (1948).
17. Kornberg, A., and Pricer, W. E., *ibid.*, **191**, 535 (1951).
18. Lehninger, A. L., and Smith, S. W., *ibid.*, **181**, 415 (1949).
19. Lindberg, O., and Hummel, J. P., *Arkiv Kemi*, **1**, 17 (1949).
20. Martin, J. M., and Doty, D. M., *Anal. Chem.*, **21**, 965 (1949).
21. Niemeyer, H., Crane, R. K., Kennedy, E. P., and Lipmann, F., *Federation Proc.*, **10**, 229 (1951).
22. Potter, V. R., *Ann. Rev. Bioch.*, **XIX**, 1 (1950).
23. Slater, E. C., *Nature*, **166**, 982 (1950).
24. Teply, L. J., *Arch.Biochem.*, **24**, 383 (1949).
25. Zetterström, R., Ernster, L., and Lindberg, O., *Arch. Biochem. et Biophys.*, **31**, 113 (1951 .
26. Zetterström, R., and Ljunggren, M., *Acta Chem. Scand.*, **5**, 291 (1951).

BRIEF NOTES

THE QUANTITATIVE ESTIMATION OF DESOXYRIBOSE NUCLEIC ACID (DNA) IN ISOLATED INDIVIDUAL ANIMAL NUCLEI BY THE CASPERSSON ULTRAVIOLET METHOD

CECILIE LEUCHTENBERGER[1], RUDOLF LEUCHTENBERGER[2], COLETTE VENDRELY[3], and ROGER VENDRELY[4]

Department of Cell Research, Karolinska Institutet, Stockholm, Sweden

Received October 1, 1951

The development of an Ultraviolet Microscopic Spectrophotometric method by Caspersson (2) in 1936 made it possible to analyze *individual* cells and cell parts *in situ* for their chemical components. This approach not only contributed fundamentally to our knowledge on such important intracellular substances as nucleic acids (3), but also stimulated the development of microscopic photometric methods in other spectral ranges (6, 10).

But in spite of the obvious advantages and possibilities of such cytochemical procedures which allow the direct correlation between structures and chemical composition, the number of biological objects suitable for microspectrophotometry is rather restricted (3). One serious handicap for quantitative microspectrophotometric work on a biological specimen is the inhomogeneous distribution of its absorbing material and whereas some methods, such as freeze-drying, seem to reduce the inhomogeneity of intracellular components (3), the problem is by no means solved. The present report describes material which in view of its relative homogeneous distribution of absorbing substances and due to its chemical composition seems suited for a quantitative estimation of DNA by means of the Caspersson microspectrophotometric method. The values of the cytochemical analysis of absolute DNA amounts in individual nuclei and in sperms are in close agreement with those of the chemical determinations obtained on the same material.

Material and methods: Liver, thymus and sperm were obtained from freshly killed beef and rat, the nuclei isolated and the amount of DNA determined as previously described (11). For the cytochemical analysis a mixture of the same isolated nuclei and sperms was suspended for one hour in glycerine, and a drop,

[1] Present address: Institute of Pathology, Western Reserve University, Cleveland 6, Ohio, U.S.A.
[2] Doctors Hospital, Department of Pathology, Cleveland 6, Ohio, U.S.A.
[3] Institut d'Embryologie, Université de Strasbourg, France.
[4] Institut de Bactériologie, Université de Strasbourg, France.

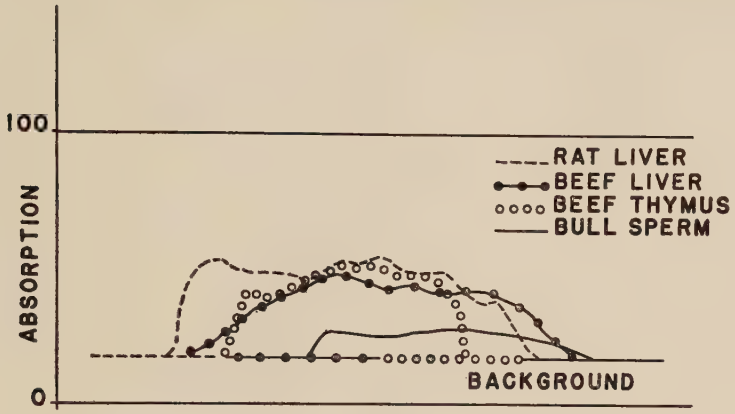


Figure 1. Curves made with a recording densitometer across photographs of nuclei and sperm taken at 2 570 Å.

of the suspension put on a quartzslide. The number of nuclei and sperm should be rather small in relation to the amount of glycerine, so that on the quartzslide there is a large free space present adjacent to the nucleus or sperm. Such a technique eliminates errors which might arise from possible inhomogeneity in the emulsion of the photographic plate or inhomogeneous illumination. The absorption measurements were carried out according to the photographic microspectrophotometric method of Caspersson (2). The amount of DNA per nucleus was computed according to the following formula

$$\text{DNA} = \frac{A \times E \text{ mg}}{\varepsilon}$$

where A = Area of nucleus, E = Extinction and $\varepsilon = 20$, corresponding to the computed extinction of a standard solution of DNA of 1 mg per 1 cc^3 in a 1 cm path (7). The area of the nuclei and sperms were measured with a planimeter from direct enlargements of photographs of these objects.

Results: The cytological appearance of bull sperms in glycerine at 2 570 Å shows a rather striking uniformity of the absorbing material, in spite of the somewhat denser posterior and somewhat lighter central portion, which incidentally parallels Bretschneider's (1) electron microscopic studies of bull sperms. The distribution of the absorbing substances in the isolated nuclei, although by far not as homogeneous as that of the sperm, is nevertheless relatively uniform. It reminds somewhat of the uniform appearance of living nuclei as seen under the phase microscope. Curves made with a recording densitometer across photographs of sperms and nuclei reflect the homogeneity rather well, as can be seen from Fig. 1. In order to compute the DNA amounts for nuclei and sperms from the absorption data at 2 570 Å, it was necessary to make absorption *curves* of the same material at different wave-lengths.

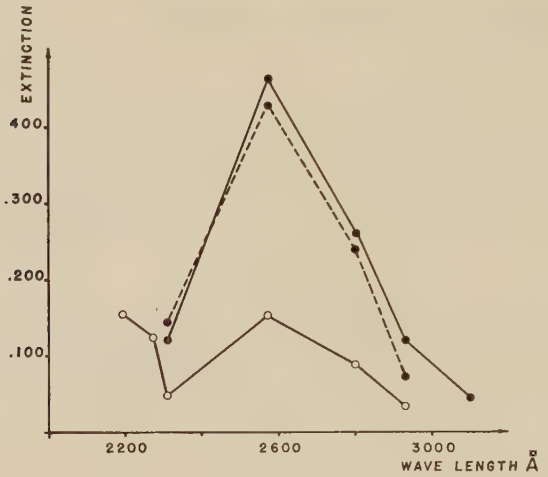


Figure 2. Absorption spectra of individual liver nuclei (• — • Rat Liver) (• ··· • Beef Liver) (○—○ Bull Sperm).

TABLE I

Determination of the amounts of DNA in isolated nuclei of different tissues of beef and rat and of bull sperm by cytochemical (Caspersson u.v. method) and chemical analysis.

Tissue and no. of nuclei measured	Cytochemical analysis Mean amt. of DNA per nucleus in 10^{-9} mg	Chemical analysis		
		Mean amt. of DNA per nucleus in 10^{-9} mg	Mean amt. of RNA per nucleus in 10^{-9} mg	Mean amt. of proteins per nucleus in 10^{-9} mg
Beef liver (42)	5.9±0.11	6.4	0.3	20.8
Beef thymus (16[1])	5.7±0.22	6.4	0.5	14.3
Bull sperm (56).....	2.8±0.03	3.3	0.1	14.0
Rat liver (47)	6.0±0.40	8.6	—	—
	10.5±0.20			
	19.1±0.67			

An example of such curves is given in Fig. 2. It is evident that these curves resemble those of pure nucleic acid with a peak at 2 570 Å and a ratio between 2 800 Å and 2 570 Å of approximately 0.6. The non-interference of protein absorption at 2 570 Å is easily understood, if one considers that the ratio between proteins and DNA in this material varies from 2–4 : 1 (see Table I, columns 3 and 5). Since the absorption at 2 570 Å of nucleic acid groups is about 50 times that of a protein solution

[1] The number of thymus nuclei is small, because due to the small size and high concentration of DNA of most thymus nuclei, the extinctions at 2 570 Å were extremely high, and therefore could not be used for the computations of DNA.

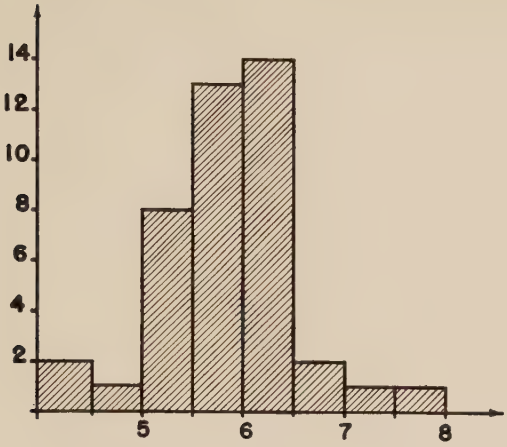


Figure 3. Amount of DNA in individual beef liver nuclei. (On vertical axis-No. of nuclei.) (On horizontal axis-DNA in 10^{-9} mg.)

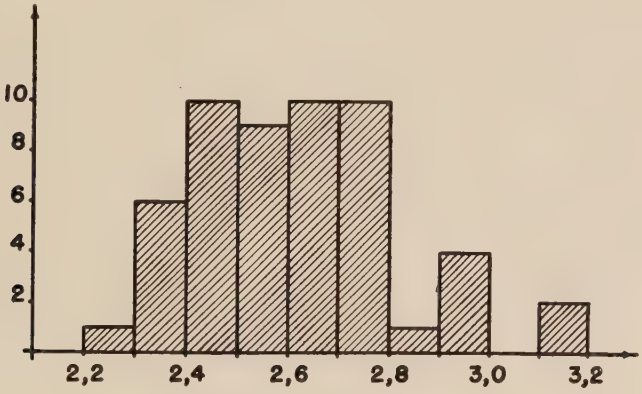


Figure 4. Amount of DNA in individual bull sperms. (On vertical axis-No. of nuclei.) (On horizontal axis-DNA in 10^{-9} mg.)

in the same concentration (4), the absorption at 2 570 Å due to proteins in this material can be neglected when computing the DNA amount. In view of the low amounts of ribosenucleic acid (RNA) in the isolated nuclei (constituting about 5 per cent of the total nucleic acids and probably due partly to cytoplasmic impurities), it seems therefore justified to compute from the absorption at 2 570 Å the amounts of DNA per nucleus and sperm. The results are presented in Table I, figs. 3 and 4.

The values presented in Table I show that if a number of individual beef liver nuclei or bull sperm are analysed for DNA by the Caspersson photographic microspectrophotometric method, the mean value is in close agreement with that obtained

by the chemical analysis. Furthermore the DNA amount of beef liver nuclei is twice that of the sperms. It may be mentioned that in spite of the rather constant DNA amount in individual nuclei the nuclear and sperm sizes may vary considerably. When comparing the chemical and cytochemical analysis of the rat liver nuclei, it can be noted that whereas cytochemically three types of nuclei are found with values of DNA per nucleus representing nearly exact multiples of each other, the value of the chemical analysis is an *average* value, resulting from lumping together the different DNA values. These observations are in accordance with previous studies, in which the DNA was estimated cytochemically by the Feulgen reaction in isolated nuclei and compared with the chemical analysis on the same material (8). The amount of DNA for isolated thymus nuclei of beef as estimated in this study by the cytochemical and chemical analysis is not in agreement with the data of Pollister and Ris (10).

In conclusion some of the advantages of using isolated nuclei instead of tissue sections for microspectrophotometric determinations of DNA are discussed:

1) The distribution of the absorbing substances in *isolated* nuclei in glycerine is much more favorable from the optical point of view than the one in tissue sections of the same material, the difference probably being due to fixatives and the embedding process used for sections of the same material.

2) The presence of a large free space directly adjacent to the nucleus for background measurements, very rarely encountered in tissue sections, is another advantage of nuclear suspensions.

3) Due to the marked loss of proteins from the nuclei by isolation procedures (8, 9) and the low amounts of ribosenucleic acid, the DNA can be estimated directly from the extinction values.

4) The analysis of *whole* nuclei instead of sectioned cells does not only simplify the quantitative estimation of DNA but also eliminates the errors arising from measurements of thickness of sections.

5) The possibility of adding a known standard —e.g. bull sperm—to a nuclear suspension to be analysed and the control of the cytochemical data by chemical analysis of the same material increases the accuracy of the results.

REFERENCES

1. Bretschneider, L. H., *Proc. Koninkl. Nederland Akad. Wetenschap.*, **LIII**, 4 (1950).
2. Caspersson, T., *Skand. Arch. f. Phys.*, **73**, Suppl. 8 (1936).
3. ——, Cell growth and cell function, Norton Co., 1950.
4. Caspersson, T., and Santesson, L., *Acta Radiologica, Suppl.* **XLVI** (1942).
5. Dounce, A. L., The Enzymes. Acad. Press N. Y., **1**, 187, 1950.
6. Engström, A., *Nature*, **158**, 664 (1946).
7. Leuchtenberger, C., *Chromosoma*, **3**, 449 (1950).
8. Leuchtenberger, C., Vendrely, R., and Vendrely, C., *Proc. Natl. Acad. Sci.*, **37**, 33 (1951).
9. Pollister, A. W., and Leuchtenberger, C., *ibid*, **35**, 66 (1949).
10. Pollister, A. W., and Ris, H., *Cold Spring Harbor Symposia Quant. Biol.*, **XII**, 147 (1947).
11. Vendrely, R., and Vendrely, C., *Experientia*, **IV**, 434 (1948).

BIOLOGIC EFFECTS OF X-IRRADIATION[1]

L. C. FOGG and R. F. COWING

Pondville Hospital, P. O. Walpole, Mass., and Massachusetts Department of Public Health

Received October 17, 1951

The submaxillary gland in mice and rats shows sex dimorphism, as has been demonstrated by Lacassagne (6), Fekete (1) and Frantz and Kirschbaum (3). This gland can be altered either by injection of androgens into the ovariectomized female (3) or by castration of the male. Thus by the use of proper procedures the dimorphism is capable of reversal from male to female type or female to male type.

It is not known what cells in the gonads are associated with this dimorphism. Selective destruction of germ cells and Sertoli cells in testicular tissue by acute x-ray, however, provides a method for determining whether germinal epithelium is involved. That the germinal epithelium may have an endocrine function or secrete a hormone has been suggested in the literature. Liebow, Warren and DeCoursey (8) suggested that germinal epithelium or its derivatives may have an endocrine function. Their thesis is based on the report of Joel (5) that "castration cells" are found in the pituitary following irradiation of the testes. Howard, Sniffen, Simmons, and Albright (4) believe that the presence of "castration cells" suggests the possibility that germinal cells secrete a hormone. It is further reported by LeRoy (7) that "castration cells" appeared in the pituitary as sequelae in cases resulting from the atomic bomb explosions in Nagasaki and Hiroshima.

It has been demonstrated that an acute dose of 2 000r x-irradiation will eventually result in the loss of all the germ cells in the testis of the pure line C57 or C3H mouse without concomitant morphological change in the Sertoli cells or the interstitial cells (2). A dose of 4 500r in the Sloniker strain rat produces a loss of the Sertoli cells as well as the germinal cells but leaves the interstitial cells intact.

For comparison a set of 14 mice, of C3H and C57 strains and 2 Sloniker rats, were castrated by excision through the scrotal sac. The submaxillary glands of both the x-irradiated and of the castrated animals were examined microscopically at intervals post irradiation or post castration.

Results: The normal male submaxillary gland is characterized by the presence of varying amounts of granules in the distal part of the cells in the terminal tubule (Fig. 1). The female gland lacks these granules (Fig. 2).

The gland of the castrated male became feminized in a period of four to six weeks (Fig. 3.)

[1] This work was supported by Grant C-1047 from the United States Public Health Service and by the Department of Public Health, Pondville Hospital.

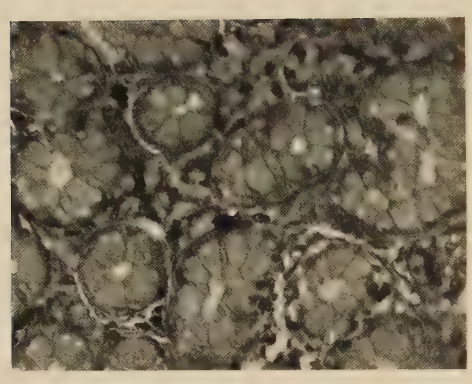


Fig. 1.

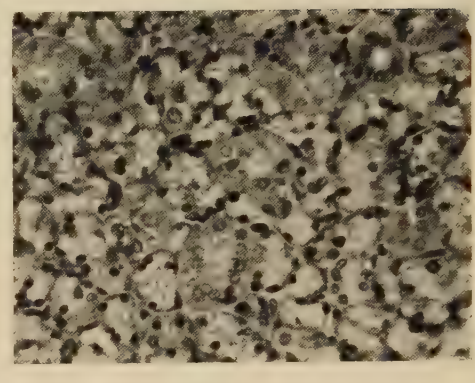


Fig. 2.

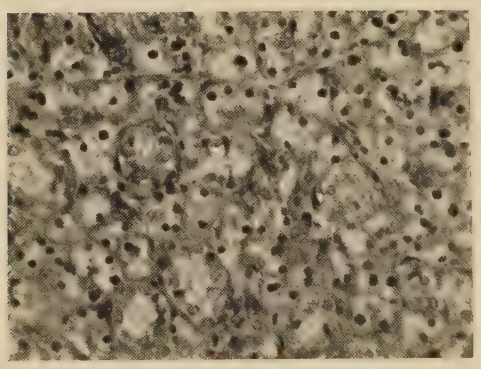


Fig. 3.

Fig. 1. Section of typical submaxillary gland from normal C3H male mouse (×500). Hematoxylin and eosin.

Fig. 2. Section of typical submaxillary gland from normal C3H female mouse (×500). Hematoxylin and eosin.

Fig. 3. Appearance of submaxillary gland of C57 mouse 44 days post castration (×500). Gland feminized.

At a dose of 2000r, 42 days after direct x-irradiation to the testes, the testicular tubules were characterized by the absence of germ cells, but the Sertoli and interstitial cells were present and appeared normal morphologically. This agerminal state did not cause feminization of the male submaxillary gland.

It remained to be determined if the Sertoli cells were involved in the submaxillary changes. Direct x-irradiation to the rat testes (4500r, 100 KvP, 15 Ma, 2.6 mm. Al, Hvl, 187r/minute at TSD of 20 cm.) caused eventual destruction of both the germinal cells and the Sertoli cells but left other tissue intact. Under this condition, the male submaxillary gland did not become feminized, thus eliminating the Sertoli cells as a factor contributing to dimorphism of the gland.

It also remained to be determined if castration following direct x-irradiation had any influence on the castration effect. The experimental data are submitted in Table I.

It can be concluded that a castrated irradiated animal and castration in absence of irradiation have the same effect as concerns induction of feminization of the male submaxillary gland.

TABLE I

Direct irradiation plus Castration 1440r

Days		Sacrificed Post-Castration	Number of Animals	State of Submaxillary
Irradiated	Castrated Post Irradiation			
0	5 days	7 days	4	NF
0	5 days	28 days	4	I
0	5 days	18 days	4	NF
0	5 days	42 days	4	F
0	7 days	35 days	4	I
0	6 days	28 days	4	I
0	15 days	27 days	2	I
0	21 days	7 days	4	NF
0	21 days	14 days	4	I
0	21 days	28 days	4	I

NF = Not feminized.
I = Intermediate.
F = Feminized.

Summary: Direct x-irradiation of testes of mice with 2 000r and of rats with 4 500r results in the loss of germinal cells alone or germinal cells and Sertoli cells without apparent morphological change of the interstitial cells. The induced sterility does not feminize the submaxillary gland as does castration. No additive effect was noted when castration after acute x-irradiation was the method of treatment.

REFERENCES

1. FEKETE, E., Biology of the Laboratory Mouse. p. 112, 1941.
2. FOGG, L., and COWING, R., *Cancer Res.*, **1**, 23 (1951).
3. FRANTZ, M. J., and KIRSCHBAUM, A., *ibid*, **9**, 257 (1949).
4. HOWARD, R. P., SNIFFEN, R. C., SIMMONS, F. A., and ALBRIGHT, F., *J. Clin. Endocrinol.*, **10**, 121 (1950).
5 JOEL, C. A., *Schweiz. Med. Wochschr.*, **23**, 795 (1942).
6. LACASSAGNE, A., *Compt. Rend. Soc. Biol.*, **133**, (1940).
7. LEROY, G. V., *J. Am. Med. Ass.*, **134**, 1143 (1947).
8. LIEBOW, A., WARREN, S., DECOURSEY, E., *Am. J. Path.*, **25**, 919, 924, 928 (1949).
9. RAYNAUD, J., *Ann. Endocrinol. (Paris)*, **8**, 359 (1947).

DETERMINATION BY INTERFEROMETRIC METHODS OF THE THICKNESS OF THE REFERENCE SYSTEM USED IN MICRORADIOGRAPHY

O. HALLÉN and E. INGELSTAM

Department of Histology, Medical School, Göteborg, Sweden and The Optics Laboratory, Royal Institute of Technology, Stockholm, Sweden

Received October 30, 1951

An investigation regarding the importance of the reference system used in microradiography for determination of mass in biological structures has been reported (1). In that paper the essential factors for the formation of a nitrocellulose surface film were discussed from a physicochemical viewpoint. A new method and apparatus for the preparation of the film were described. Gravimetric methods were used in order to obtain the mass per unit area of the film. Compared with earlier methods the method described has greater accuracy, involves fewer sources of error in the reference system, and simplifies the manual procedure.

At the Optics Laboratory of the Royal Institute of Technology, Stockholm, a preliminary investigation has been made to elaborate optical methods for the determination of mass per unit area in the different steps of the wedge, forming the reference system. By means of interferometric methods measuring thicknesses or optical paths it was possible to increase the accuracy of these measurements.

The most accurate measurements have been performed with multiple beam interference in a reflected Fizeau arrangement. The film to be measured, bordered by an edge, is placed in the narrow space between highly reflecting plane surfaces, either touching or not touching them. The thickness is obtained as a displacement of very narrow fringes viewed in a microscope, with an accuracy of about 0.002 microns (2). As the step does not only contain distances measurable in the interferogram, but some additional distinct number of fringes, i.e. a number of half a light wavelength, it is more practical to work with at least two different wavelengths in order to find this number. When the measurement was extended over about 2 mm square of the film, the examination of a specimen gave 1 410 microns (Na 5 896 and 5 890 A), 1 404 microns (Hg 5 461 A), and 1 404 (Hg 4 358 A). The main difference in the last decimal place was due to the lacking uniformity in the film. The method makes a very exact determination of film uniformity possible.

Another method, probably still more rapid but inferior in precision, is to use the displacement of the zero order interference fringe in a Young double slit system, when one of the slits is covered by the film. Measurements made hitherto indicate the suitability of this method, which gave a precision of a few per cent.

REFERENCES

1. Brattgård, S.-O., and Hallén, O., *Biochim. et Biophys. Acta.* (In press).
2. Ingelstam, E., *Repts. Optics Lab. Roy. Inst. Technol., Stockholm*, Nr. 14 (1951).

DIE CHEMISCHEN EIGENSCHAFTEN DER NEUROSEKRETORISCHEN SUBSTANZ IN HYPOTHALAMUS UND NEUROHYPOPHYSE

THEODOR HEINRICH SCHIEBLER

Anatomisches Institut der Universität, Kiel, Deutschland, und Institut für Zellforschung, Karolinska Institutet, Stockholm, Schweden

Eingegangen am 25. Juli 1951

Bargmann und Mitarbeiter (2) wiesen in den Ganglienzellen des Nucleus supraopticus und paraventricularis hypothalami sowie im Tractus supraoptico-hypophyseus und in der Neurohypophyse aller Wirbeltierklassen eine mittels einer Chromhämatoxylinfärbung von Gomori elektiv anfärbbare, neurosekretorische Substanz nach. Diese Substanz wird nach Bargmann (1) und Hild (4) in den erwähnten Hirnarealen gebildet und zur Neurohypophyse abgeleitet. Sie soll auf Grund tierexperimenteller und pharmokologischer Untersuchungen (1, 5, 9) eine Trägersubstanz der sog. „Hypophysenhinterlappenhormone" darstellen. Die Hormone selbst werden nach ihrem Entstehungsort besser als Hypothalamushormone bezeichnet. Um nun die chemischen Eigenschaften des Neurosekretes aufzuklären, führten wir eine systematische, cytochemische Untersuchung durch. Wir verwendeten lebensfrisch fixierte Hypothalamus-Hypophysenblöcke von Menschen, Hunden, Katzen, Kaninchen, Rindern, Ratten und Hechten.

Das Neurosekret ist bei allen untersuchten Lebewesen nur dann nachzuweisen, wenn alkoholhaltige Fixative vermieden werden. Die neurosekretorische Substanz wird nämlich aus unfixiertem Gewebe durch alle fettlösende Chemikalien (Alkohol, Aceton, Äther, Benzol u. a.) extrahiert. Nach der Extraktion ist sie dann *in vitro* färberisch nachweisbar (5). Werden die fettlösenden Mittel jedoch nach der Fixierung — besonders geeignet sind Susa nach Heidenhain und Bouin'sches Fixativ — angewendet, extrahieren sie das Neurosekret nicht mehr. Die elektive Färbbarkeit des Neurosekretes ist von einer Oxydation abhängig, die der Färbung vorausgeschickt wird. Zur Färbung selbst sind eine grössere Anzahl von Kernfarbstoffen (Hämatoxyline, Gallaminblau, Thionin u. a.) geeignet. Oxydation und Reduktion spielen anscheinend für den Stoffwechsel des Neurosekretes eine besondere Rolle. Hierfür spricht auch der positive Ausfall der Oxydationsreaktion von Giroud-Leblond.

Dort wo Sekretgranula mit Chromhämatoxylin darstellbar sind, sehen wir eine positive Fettreaktion (Sudanschwarz B, Scharlachrot). So sind die Nervenzellen des Nucleus supraopticus und paraventricularis, die strotzend mit sudanophilen Granula beladen sind, mit Neurosekret angefüllt. Die sekretfreien Zellen sind dagegen praktisch fettfrei. Eine Sonderstellung nehmen die Nervenzellen ein, die gerade Neurosekret neubilden. Sie enthalten zwar Neurosekret, sind aber fettfrei. In vielen distalen Teilen des neurosekretorischen Systems sind sudanophile und neurose-

kretorische Substanz ebenfalls gleich lokalisiert, wie z. B. in den Herringkörpern der Neurohypophyse. Diese Beobachtungen legen in Uebereinstimmung mit der Alkohollöslichkeit den Gedanken nahe, dass das Neurosekret lipoide Eigenschaften besitzt. Diese Definition dürfte jedoch noch nicht vollständig sein, da wir im Neurosekret ferner Polysaccharide und Proteine nachweisen können. Durch die Perjodsäure-Schiff (PJS) Reaktion von Hotchkiss (6) werden grosse Teile des neurosekretorischen Systems dargestellt, da die neurosekretorische Substanz und das PJS positive Material gleiche Lokalisation und Abhängigkeit von alkoholfreier Fixation aufweisen. Die PJS Reaktion fällt jedoch nicht in allen Teilen des Systems gleich intensiv aus. Die Farbintensitäten schwanken von rosa bis purpurrot. Ferner beobachten wir auch Gomori-positive, PJS-negative Stellen. Durch die reversible Azetylierungstechnik (8) können wir wahrscheinlich machen, dass Glykolgruppen für die positive PJS Reaktion im Neurosekret verantwortlich sind. Hyaluronsäure schlossen wir durch Vorbehandlung mit 500 TRU Wyeth Hyaluronidase und Glykogen durch Digestion mit Amylasen aus. Ebenfalls enthält das Neurosekret keine PJS positiven Lipoide, da durch heisses Methanol-Chloroform (3) keine Extraktion möglich ist. Auf Proteine schlossen wir aus dem positiven Ausfall der Trypsindigestion. Nach etwa 12 Stunden Digestion ist im Gewebe kein Neurosekret mehr nachweisbar. Ferner fällt die Millonreaktion (7) in grossen Teilen des neurosekretorischen Systems positiv aus. Besonders deutlich sind dabei wiederum das Zytoplasma zahlreicher neurosekrethaltiger Ganglienzellen und die Herringkörper der Neurohypophyse dargestellt. Von besonderem Interesse ist schliesslich noch die Frage nach dem Vorhandensein von Nucleinsäuren im Neurosekret. Bekanntlich nimmt die nucleinsäurehaltige Nisslsubstanz in dem Masse ab, wie Neurosekret entsteht. Unsere Untersuchungen ergeben jedoch, dass das Neurosekret nucleinsäurefrei zu sein scheint.

Wir können unsere Beobachtungen dahingehend präzisieren, dass im Neurosekret Lipoide, Kohlehydrate und Eiweisse vorhanden zu sein scheinen. Vermutlich stellt das Neurosekret einen Glykolipoproteinkomplex dar. Diese Zusammensetzung ist bei allen von uns untersuchten Tierklassen konstant. Jedoch scheint das quantitative Verhältnis der 3 Stoffgruppen zueinander auch beim einzelnen Tierindividuum erheblich zu schwanken. Bald überwiegt diese, bald jene Gruppe. Die Untersuchung über die Abhängigkeit der Zusammensetzung des Neurosekretes von der Funktion und die quantitative Auswertung sind in Vorbereitung.

LITERATUR

1. Bargmann, W., *Z. Zellforsch.*, **34**, 610 (1949).
2. Bargmann, W., Hild, W., Ortmann, R., und Schiebler, T. H., *Acta neurovegetat.*, **1**, 233 (1951).
3. Gersh, I., *Arch. Path.*, **47**, 99 (1949).
4. Hild, W., *Virchow's Arch.*, **319**, 526 (1951).
5. Hild, W., und Zetler, G., *Arch. exptl. Path. u. Pharmakol.*, **213**, 139 (1951).
6. Hotchkiss, R. D., *Arch. Biochem.*, **16**, 131 (1948).
7. Leuchtenberger, C., *Chromosoma*, **3**, 449 (1950).
8. McManus, J. und Cason, J., *J. Exptl. Med.*, **91**, 651 (1950).
9. Ortmann, R., *Z. Zellforsch.*, **36**, 92 (1951).